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THE ANTAI-INFLAMMATORY EFFICACY AND ADRENAL SUPPRESSIVE
EFFECTS OF COLON-SPECIFIC GLUCOCORTICOID PRODRUGS ON
EXPERIMENTAL COLITIS

BY

NINGREN CUI ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE
IN
EXPERIMENTAL MEDICINE

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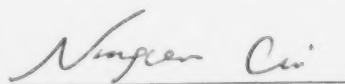
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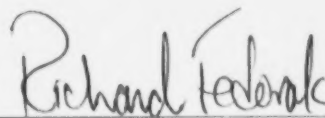


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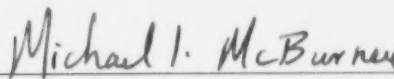
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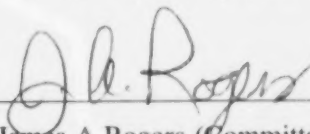
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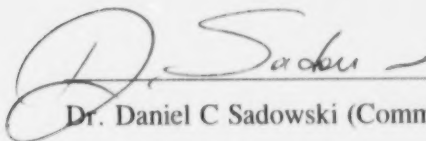
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DEDICATION

TO MY PARENTS FOR THEIR LOVE AND SUPPORT.

TO MY WIFE AND DAUGHTER FOR THEIR LOVE AND SACRIFICE.

ABSTRACT

Although oral glucocorticoids are the treatment of choice for moderate to severe inflammatory bowel disease (IBD), their severe side-effects and hypothalamic-pituitary-adrenal suppression after systemic absorption limit application of these drugs. Newly synthesized glucocorticoid prodrugs (budesonide- β -D-glucuronide, dexamethasone-succinate-dextran, dexamethasone-glutarate-dexamethasone, methylprednisolone-succinate-dextran, and dexamethasone- β -D-glucuronide) are not absorbed or hydrolyzed in the small intestine, but are hydrolyzed in the large intestine by bacterial enzymes. The free anti-inflammatory agents are released in the colon and act locally. In this study distribution of free dexamethasone and dexamethasone- β -D-glucuronide in the gastrointestinal (GI) tract and absorption into blood stream were examined in normal rats after a single dose. Colitis was induced by instillation of 4% acetic acid into the rat colon. Twenty-four to forty-eight hours after the induction of colitis, free glucocorticoids and their prodrugs were administered by oral gavage. Seventy-two hours after the induction of colitis the efficacies of the prodrugs were determined by measuring *in vivo* colonic fluid absorption, colonic macroscopic ulceration, and myeloperoxidase activity. Adrenal suppressive effects of the prodrugs were determined by measuring serum corticosterone and plasma adrenocorticotrophic hormone (ACTH). The distribution study revealed that 96% of dexamethasone- β -D-glucuronide was recovered in the colon, compared to only 15% of free dexamethasone. Blood concentrations of dexamethasone in rats treated with free dexamethasone were 3-fold higher than in rats treated with dexamethasone- β -D-glucuronide. Treatment with oral prodrugs accelerated colitis healing at significantly lower doses than free drugs. Treatment with prodrugs avoided hypothalamic-pituitary-adrenal suppression with most of the doses whereas it was significant after treatment with free drugs. Development of orally-administered colon-specific glucocorticoid delivery systems represents a promising approach to IBD treatment.

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LIST OF ABBREVIATIONS

IBD	inflammatory bowel disease
UC	ulcerative colitis
CD	Crohn's disease
DX	dexamethasone
BD	budesonide
MP	methylprednisolone
DX-glrd	dexamethasone- β -D-glucuronide
BD-glrd	budesonide- β -D-glucuronide
DS-dextran	dexamethasone-succinate-dextran
DG-dextran	dexamethasone-glutarate-dextran
MPS-dextran	methylprednisolone-succinate-dextran
5-ASA	5-aminosalicylic acid
ACTH	adrenocorticotrophic hormone
AAC	acetic acid
MPO	myeloperoxidase
ED ₅₀	the dose for 50% of maximal effect
HPLC	high performance liquid chromatography
RIA	radioimmunoassay
SD	standard deviation
GI	gastrointestinal
PG	prostaglandin
LT	leukotriene
IL	interleukin
TNF	tumor necrosis factor
IFN	interferon

CHAPTER 1

INTRODUCTION

Inflammatory bowel disease (IBD) is common in Europe and North America. This morbid process includes Crohn's disease (CD) and ulcerative colitis (UC). Although information about the disease has been rapidly accumulating, the etiology of IBD is unknown and current treatment modalities of the disease are not satisfactory.

IBD severely impairs a person's quality of life. Onset occurs when patients are young and the disease recurs life-long. It results in mortality in 20% of its victims within 20 years of onset. The cost for treatment of the disease totals one-tenth of the entire amount spent on all gastrointestinal diseases. The incidence of the disease has been increasing in the last thirty years.

Anti-inflammatory drug therapy for IBD (steroidal and non-steroidal anti-inflammatory drugs) is effective, but is accompanied by numerous, sometimes severe, systemic side-effects. In order to increase the efficacy and reduce the side-effects of the drugs, a number of colon-specific prodrugs from which the active compounds are only released in the large intestine by bacterial enzymes, have been developed. The ideal colon-specific delivery of glucocorticoid prodrugs would involve a prodrug stable in the upper GI tract, releasing the drug only in the caecum and colon. The hypothesis of this study is that administration of this type of prodrug would lead to a lower free drug concentration in the blood and a higher free drug concentration in colonic luminal contents and mucosa. Thus, the ideal prodrug would increase the efficacy and decrease the side-effects of the drug.

Based on this hypothesis, the efficacy and adrenal suppressive effects of a number of glucocorticoid prodrugs on an acetic acid-induced rat colitis model were determined. Distribution studies on one of the glucocorticoid prodrugs, dexamethasone- β -D-glucuronide (DX-glrd), was also performed to test the release and absorption of the free dexamethasone (DX) in the gastrointestinal (GI) tract.

This thesis is organized in the following manner. In chapter 2, the current background knowledge about IBD, with an emphasis on the mechanisms of tissue injury is introduced. In chapter 3, the current background knowledge about glucocorticoids is reviewed. This review focuses on the mechanisms by which glucocorticoids exert their anti-inflammatory actions and side-effects. Also progress in the production of colon-specific glucocorticoid prodrugs since the early 1980's is updated. Chapter 4 examines the efficacy of the prodrugs by measuring in vivo colonic fluid absorption, macroscopic ulceration, and myeloperoxidase activity. Chapter 5 examines the distribution of DX-glrd and free DX by comparing the concentrations of active compound to be found in the contents of the different intestinal segments and in the blood after oral administration of either the prodrug or its free parent drug. Then adrenal suppressive effects of the prodrugs are tested by measuring serum corticosterone and plasma ACTH levels. Finally, the results are summarized and the future research directions are discussed in Chapter 6.

CHAPTER 2

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) includes Crohn's disease (CD), which may involve any part of the GI tract, and ulcerative colitis (UC), which only involves the colon. Although first reported in 1793 (1) and recognized as a distinct pathological and clinical entity in 1932 (2), IBD still remains one of the great challenges of gastroenterology.

2.1 ETIOLOGY

The etiology of IBD is still under investigation. Different theories have proposed the involvement of genetic factors, immune deficiencies, bacterial infections, and arachidonic acid metabolites. Each of these theories explains the etiology of some aspects of IBD, but no single general explanation exists.

2.1.1 Genetic factors

It is believed that genetic factors play a role in the etiology of IBD. The incidence of IBD is highest among Caucasians, lower in blacks, and lowest in Asians. Mayberry et al reported that the prevalence of the disease among siblings was about 30-fold that of the community-wide rate (3). There is a higher concordance rate in twins than in the community-wide population (4). The offspring of parents who are both affected by the disease have higher risk of developing IBD (5). Genetic marker studies are one approach to the identification of the actual genes that predispose a person to IBD. Some connections have been found between IBD and human lymphocyte antigen (HLA) markers. One study involved a Caucasian population and a carefully selected, ethnically-matched control group. The HLA-DR2 marker was found to occur with increased frequency in UC patients (6,7). Tiwari et al speculated that the increased frequency of DR2 occurrence might provide important clues to the etiology of IBD, as this antigen had been associated with an increased susceptibility to infection and multiple sclerosis (8). One study from

Holland reported that HLA-B18 was significantly increased in CD patients (9). Another study from Japan found a dramatic increase in HLA-DR4 in CD patients (10). Other studies have shown an increased intestinal permeability in patients with CD (11,12). Interestingly, intestinal permeability is also increased in healthy relatives of patients with CD as compared to the intestinal permeability of healthy controls (13). These findings indicate that this defect in permeability could be potentially used as a sub-clinical marker.

2.1.2 Immune disorders

The immune system is also thought to play a role in the etiology of IBD. One phenomenon indicating the disturbance of the immune system in patients with IBD is the production of auto-antibodies (14,15). These auto-antibodies react against various intestinal epithelial antigens, as well as against extra-intestinal antigens (16, 17). Another autoantibody, antineutrophil cytoplasmic antibody (ANCA), is found in 70% UC patients (18). It is also reported that 16% of clinically healthy relatives of UC patients are ANCA positive compared with 3% of environmental controls (19). The activation of the immune system in cases of IBD involves many of the cell systems in the GI tract. Macrophages are activated (14) and the activated macrophages release cytokines (20), arachidonic acid metabolites (21), lysosomal enzymes (22) and free oxygen radicals (23). They also present antigens /class II major histocompatibility complex (class II MHC) which activate helper T cells (24). The activated helper T cells secrete a number of cytokines including interleukins, tumour necrosis factors (TNF- α , β), and interferon γ (IFN- γ); these cytokines in turn activate cytotoxic T cells, mast cells, and B cells. In addition to cytokines, the activated macrophages also release inflammatory mediators (leukotrienes [LTs], superoxides, prostaglandins [PGs]) which damage tissues (25,26). It is known that interleukin-1(IL-1) is mainly produced by macrophages because removal of macrophages from cell cultures leads to significant decrease of IL-1 production (27). IL-1 levels are elevated in active ulcerative colitis and Crohn's disease and return to control levels in terms of clinical remission (27). In Crohn's disease and ulcerative colitis, TNF- α is increased in mucosal biopsy findings (28). TNF- α transcripts in lamina propria

mononuclear cells from Crohn's disease are also increased compared to normal mucosa (29).

The activated B cells secrete immunoglobulins (30) and form complement complexes which may be associated with tissue damage. Some of the cytokines released by macrophages and T cells also attract neutrophils which immigrate into the inflamed area and release lysosomal enzymes (31) and other inflammatory mediators (32).

2.1.3 Bacteriology

The role of bacterial infection in the etiology of IBD has long been suspected (33). Mitchell and Rees injected rabbits with both ileal filtrates from patients with Crohn's disease and colonic filtrates from patients with ulcerative colitis and induced histologic changes similar to those seen in IBD (34). Keighley has reported a higher rate of organism recovery from the lymph nodes, serosa, liver, and portal blood of patients with active Crohn's disease than from patients with inactive Crohn's disease (35). Thayer et al found that 23% of their patients with Crohn's disease had a positive serology for *Mycobacterium Paratuberculosis* (36). The polymerase chain reaction for genomic DNA of *M. paratuberculosis* revealed significantly higher prevalence in tissue from Crohn's disease patients than in control tissue (37,38). The circulating level of bacterial products, including endotoxins and peptidoglycan-polysaccharide complexes, have been found to be elevated in patients with inflammatory bowel disease (39). Although these data and other findings indicate that bacterial infection plays a role in IBD, the bacteria which might cause the disease has not been isolated, and a causal relationship with disease activity has yet to be demonstrated.

2.1.4 Arachidonic acid metabolites

In IBD, the synthesis and release of inflammatory mediators (e.g. PGs and thromboxanes [TXs] through the cyclo-oxygenase pathway and LTs through lipo-oxygenase pathway) are increased (40). Among these mediators, LTB₄ may play an important role in the etiology of IBD by amplifying intestinal inflammatory reactions. In animal experiments a marked increase in LTB₄ synthesis was shown to occur early after

the initiation of the inflammatory response (41,42). It has been reported that the elevated LTB_4 synthesis lasts for 5 weeks after induction of colitis (41). In patients with IBD the synthesis of LTB_4 is also increased. Lauritsen et al studied the role of LTB_4 in the pathogenesis of IBD by examining the effects of anti-inflammatory therapy on disease severity and LTB_4 production (43). They found that, when patients with UC were treated with either 5-ASA or prednisolone, some patients responded well and others did not. The patients whose disease responded to the treatment had decreased levels of LTB_4 in their rectal dialysate, and those whose disease did not respond had unchanged LTB_4 levels. Thus, there was a significant correlation between LTB_4 levels and disease activity.

2.2 EPIDEMIOLOGY

Factors such as age, sex, race, residential area, and personal habits are believed to play important roles in the etiology of IBD. Generally, the incidence rates of IBD are highest in the Scandinavian countries and Great Britain. In Norway, for example, the incidence was 14.8/100,000 persons and in Sweden it was 12/100,000 persons in the 1980's (44,45). In North America, the incidence is also high. For example, in Spokane, Washington, the incidence was 8.8/100,000 persons in 1981 (46). In contrast, the incidence is under 1/100,000 persons in Asian countries and South Africa. Interestingly, the incidence rates are higher among persons of Jewish descent born in Europe and North America than among those of this background born in Asia and Africa (46). This discrepancy demonstrates that differences exist in and between races and regions. The incidence rate of ulcerative colitis is 3-5 times higher than that of Crohn's disease (46). The incidence of IBD has increased during the past thirty years. In Copenhagen and surrounding area, for example, it has increased from less than 1/100,000 persons to 4/100,000 persons (47). The prevalence rates in Western societies are generally 12 times higher than incidence rates (48).

The quality of life of persons suffering from IBD is significantly affected in terms of changes in systemic symptoms, bowel symptoms, functional impairment, social

impairment, and emotional function (49).

Mortality rates for IBD parallel those for incidence. Between 1980 and 1982 mortality rates for both Crohn's disease and ulcerative colitis in the United States were 0.1/100,000 persons for both men and women. The mortality rates for whites were twice those for non-whites (47).

2.3 PATHOLOGY

In IBD the pathological changes are different in UC and CD (Table 2-1) but some of the mechanisms for tissue damage may be common.

2.3.1 Neutrophil-mediated tissue injury

Active IBD is characterized by massive neutrophil infiltration into the lamina propria accompanied by extensive mucosal injury. This characteristic led to a proposal that neutrophils play an active role in mediating some of the mucosal injury (50). It is now thought two main mechanisms are involved in neutrophil-mediated tissue injury: O_2^- -dependent and O_2^- -independent processes.

1. O_2^- -dependent process: The interaction of certain pro-inflammatory mediators (LTB_4 , platelet activating factor, immune complexes, bacterial products) with specific receptors on the neutrophil plasma membrane results in a dramatic increase in oxygen consumption due to the activation of the plasma membrane-associated NADPH oxidase and the generation of large amounts of the superoxide anion radical (O_2^-) (51):



This superoxide is unstable and will spontaneously dismutate to yield hydrogen peroxide (H_2O_2) and oxygen (O_2):



Activated neutrophils also secrete myeloperoxidase (MPO) which catalyses the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid (HOCl):



The MPO- H_2O_2 -Cl system is the most potent cytotoxic aspect of the neutrophilic system.

2. O_2 -independent process: This process includes proteases, collagenases and gelatinases secreted by neutrophils. Under normal conditions these enzymes are inhibited by α_1 -antiprotease and α_2 -macroglobulin present in intestinal interstitial fluid. In case of active IBD, when the O_2 -dependent process is activated, MPO-generated HOCl inhibits the activity of these inhibitors. Protease, collagenase, and gelatinase are thus activated and cause tissue damage (52).

2.3.2 Autoimmune injury

It was first reported about 30 years ago that patients with IBD produced circulating antibodies against colonic epithelial cells (53,54). These antibodies are not specific for ulcerative colitis and are seen in cases of Crohn's disease and other gut disorders (55). They may be detected in 20-60% of IBD patients and in some of their unaffected relatives (56). Taurog et al reported that rats transgenic for human HLA-B27 develop colonic inflammation and other autoimmune disorders and this development clearly shows that a certain HLA type can predispose to autoimmune disease (57). Das et al studied a 40 Kd protein present on epithelial cells which is recognized by serum and tissue-bound IgG in patients with ulcerative colitis (58,59). It was demonstrated that the antigen for the antibody is located basolaterally on colonic enterocytes (60). Later, Halstensen et al reported that this antigen is also co-located on the apical surface of colonic enterocytes with immunoglobulin and complement components (61). In 1993, Das et al found that the 40 kD antigen is related to tropomyosin, an actin-binding cytoskeletal protein localized in the apical cytoplasm and the brush border of colonic enterocytes (62). It has been reported that 55% of symptomatic ulcerative colitis patients have circulating antibodies to a colonic extract enriched with this protein, while less than 3% of patients with Crohn's disease have these antibodies (63). The antibodies were next found to be reactive to extracts of certain strains of *E. Coli*. It was then speculated that IBD might result from an autoimmune cross-reaction with shared antigens on intestinal epithelium and intestinal bacteria. One group of investigators studied epithelial autosensitization in patients with IBD by using erythrocytes coated with the epithelial antigen as target or indicator cells

(64, 65). They found that lymphocytes from the peripheral blood and colonic lamina propria of patients with Crohn's disease and ulcerative colitis were cytotoxic to the indicator cells. The reaction was antigen specific. These findings suggest that an auto-immune response to the antigen may be pathologically significant.

2.3.3 Mucosal immune hyperactivation with local bystander injury

A T cell-mediated immune response has recently been identified in the mucosa of subjects with Crohn's disease but not in those with ulcerative colitis (66, 67). This finding shows that T cell activation in Crohn's disease is not a non-specific secondary effect due to increased antigen uptake across a damaged epithelium, but may be a primary event. In one rat colitis model, injection of peptidoglycan into the intestinal wall generated a T cell response (68). The stimulus for the inflammation in Crohn's disease is likely to be some component of the faecal stream. After the resection of diseased bowel, there is no disease activity in the neoterminal ileum of patients with Crohn's disease if this segment is bypassed. After its reconnection to the faecal stream, aphthae and inflammation develop (69). Perlmann and Broberger demonstrated that monocytes taken from the peripheral blood of children with ulcerative colitis killed human fetal colon cells, while mononuclear cells from control subjects did not exert any cytotoxic activity (70). Shorter and co-workers found that peripheral-blood mononuclear cells taken from patients with Crohn's disease, as well as from those with ulcerative colitis, were able to kill human colonic target cells. These researchers also demonstrated that the effector cells were T cells (71). Incubating control peripheral-blood mononuclear cells with an *E. Coli* lipopolysaccharide (LPS) or with the IgM fraction from the sera of patients with IBD induced cytotoxic action against colonic target cells (72). It is thought that the anticolon antibodies arm the Fc receptor-bearing killer cells in patients with IBD and thus could induce cell death via antibody-dependent cell-mediated cytotoxicity (ADCC). In IBD, mucosal T cell activation is initiated by the antigens presented by macrophages. The activated T cells can damage the epithelium by direct autoimmune attack, or they can cause tissue damage indirectly by releasing cytokines (73). The latter activity in turn, can damage tissue by increasing

mucosal permeability and damaging the barrier function of the epithelium (74), directly damaging epithelial cells (75), attracting neutrophils which infiltrate into local tissue and release superoxidants and harmful enzymes (76, 77), activating B cells to produce antibodies and form complement complexes (78), and activating mast cells to release histamine (79). Cell-mediated immunity in the mucosa and the production of cytokines such as interferon- γ can cause mucosal ulceration and increase epithelial permeability in model systems (73, 80). In patients with Crohn's disease, serum interleukin-2 (IL-2) levels are increased (81). In the rat colitis model, IL-2 production in the colonic tissue is also increased (82). It has now been found that intestinal epithelial cells themselves can also present antigens (83). This discovery indicates that epithelial cells can activate T cells and cause epithelial damage. In IBD, intestinal mononuclear cells exhibit a decreased IgA production and an increased IgG production (84). IgA prevents the microbial pathogen from attaching themselves to mucosal epithelial cells, but it does not activate complement or inflammatory cells (85).

There is some evidence that antigens injected into humans become bound to circulating IgA and are then cleared in the liver via a secretory component-mediated transport process (86). Overall IgA secretion is decreased in IBD (87). Because the integrity and function of the mucosal immune system are critically dependent on complex regulatory events that sustain a continuous IgA-mediated immune response, the decrease in IgA seen in IBD may lead to decreased antigen exclusion and, therefore, decreased host protection by the mucosal immune response. Put another way, the decreased IgA production leads to a mucosal defense deficiency caused by an inadequate dimeric IgA response. However, when compared with normal control intestinal mononuclear cells (MNC), a marked increase in IgG secretion from the MNC of IBD is noticeable (88). The greatest increase in spontaneous IgG secretion is seen with intestinal MNC in ulcerative colitis (88). The increase in IgG is concomitant with an increase in the case of IgG-containing cells in both Crohn's disease and ulcerative colitis (89,90). The increased IgG production in IBD may represent an inappropriate immune response that could partially

include a chronic infectious process (91), because an increased IgG production might lead to an IgG1-mediated response in UC and an IgG2-mediated response in Crohn's disease and could cause tissue damage (87,88). In ulcerative colitis, there is extensive complement activation and deposition of complement components on epithelium and mucosal and submucosal vessels (61,92,93). The possibility that intestinal immunoglobulin secretion is important in the IBD process is supported by the observation that 5-ASA inhibits immunoglobulin secretion by stimulating peripheral-blood B cells (94).

2.3.4 Intestinal macrophages

Intestinal macrophages account for 10%-20% of the total cell numbers in the lamina propria. The majority of mucosal macrophages are located in the subepithelial region. Joel and colleagues demonstrated the active phagocytosis of macrophages by giving carbon particles to mice orally then identifying the particles in the macrophages of the subepithelial area of the Peyer's patch (95,96). Under physiologic conditions, the intestinal macrophage population is maintained by migration of monocytes. The reason for this migration involves the chemotactic signals generated by low-level inflammatory activity associated with exposure of the mucosal surface to dietary and bacterial antigens. In IBD, the turnover of intestinal macrophages is increased (97), and the macrophages rapidly accumulated in the inflamed area (98). The activated macrophages present antigens to T cells and activate them thus initiating cellular and humoral immune responses (99). They also directly damage tissue by releasing cytokines, superoxidants, and a number of other enzymes (100).

2.3.5 LTB₄

LTB₄ can be released upon contact with bacterial products or dietary antigen by macrophages, neutrophils, and other cells in the GI tract. As a chemotactic agent, LTB₄ attracts neutrophils from blood vessels to inflamed tissue (101), stimulate the adhesion of neutrophils to epithelial cells, and stimulates the release of cytotoxic secretions from neutrophils via a specific high affinity receptor-site (102,103). At higher concentrations,

LTB₄ induces enzyme release, probably through a low affinity receptor (104). The migration of neutrophils through the endothelial cells of blood vessels and the epithelial cells will increase intestinal permeability and allow more antigens into the cells. The superoxidants released from neutrophils also damage tissue directly.

The mechanisms of tissue injury are summarized in Figure 2-1.

2.4 SYMPTOMS

2.4.1 Systemic symptoms

Systemic symptoms of IBD include fever and leukocytosis. A fever is generated by the host defense mechanism, resulting in an enhanced IL-1 activation of T cells and an increase in antibody formation by B cells, therefore eliminating some pathogenic organisms (105). IL-6 has also been found to induce fever (106). Leukocytosis is caused by colony-stimulating factors (CSF) which are produced by activated T cells and stimulate differentiation of neutrophils, eosinophils and monocytes/macrophages from bone marrow precursors (107).

2.4.2 Diarrhea

The diarrhea of IBD is caused by two major pathophysiological changes. The first one involves the decreased absorption of water and electrolytes resulting from 1) inflammation-induced mucosal injury, 2) increased luminal osmolar load due to nutrient malabsorption, 3) rapid intestinal transit time secondary to increased propulsive motor activity, and 4) neurally and hormonally induced inhibition of sodium and chloride absorption. The second change involves increased net secretion of water and electrolytes which is the result of 1) elaborated immune- and inflammation-derived secretagogues, and 2) loss of mucosal barrier function (108).

2.4.3 Pain

Abdominal pain is a common feature of IBD and contributes significantly to the morbidity of Crohn's disease and ulcerative colitis (109,110). Pain can be acute and/or chronic and may arise from the inflammatory process involving the gastrointestinal tract,

from secondary complications, and/or from extra-intestinal manifestation of the disease.

2.5 NATURAL COURSE OF IBD

2.5.1 Ulcerative colitis

The mean interval between the onset of symptoms and diagnosis has not changed for three decades. The mean time between onset and diagnosis is 1.8 years, and the mean time for diagnosis is 5 months (111). Based on bowel movement, the amount of blood present in the stool, fever, and red cell sedimentation rate, Truelove and Witts classified the attacks of UC as mild, moderate, or severe (112). The incidence of ulcerative pancolitis is decreasing, and the incidence of ulcerative proctitis (left-side disease) is increasing (113,114). The outcome of the disease depends on the severity of the first attack and age of the patient. Edwards and Truelove followed 250 patients who had had a first attack (observation time ranging from 1 year to 24 years). They found that 54% of the patients had mild disease, 27% had moderate disease, and 19% had severe disease (115). Twenty-five patients died in the first attack, and 75% of the patients who died had severe disease. The fatality rate was higher in patients older than 60 years. The numbers of patients who had a first attack and underwent colectomy varied. One report suggested this number was 0.8% and another reported it to be 11.3% (115,116). The greatest risk of colectomy is within the first 1-2 years after diagnosis. Although 88% of the patients who suffer the first attack will enter remission, most of them will have recurrence (117). One study found that 80% of the patients had a second attack within one year after entering remission (118). Sinclair et al reported that, five years following the first attack, 12% of patients had experienced extension of the disease, and this number increased to 30% 10 years after the first attack. During a period of 20 years, the mortality rate for patients with UC was 35% (117). Another report indicated that in northeastern Scotland, the overall survival rate of UC patients was 80% during a 10 year period (118).

2.5.2 Crohn's disease

In Crohn's disease, the initial anatomical involvement is thought to be the major

determinant in the clinic course. Farmer et al have reported the results of a 10 year study which found that, in all clinical patterns, ileocolonic involvement (distal small intestine and right colon) was 41% and operations were required for 73% of these patients (119). Patients with small intestinal involvement only or colonic involvement only had a lower operation rate (51%). Others have reported that, 10 years after initial resection the re-operation rate varied from 24% to 43% depending on which bowel segment was initially resected (120). In a study of the long-term outcome of Crohn's disease Harper et al found that, in 139 patients with a minimum of 15 years of treatment, ileocolic disease increased from 45% to 75%, and 88% of the 139 patients had had at least one operation. The mortality rate was 7% during the period (121).

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Table 2-1. Pathologic differences between ulcerative colitis and Crohn's disease.

	Ulcerative colitis	Crohn's disease
location	entire colon/distal ileum	small intestine/colon
skip lesions	no	yes
thickened bowel wall	no	yes
strictures	no	yes
vascular congestion	yes	no
bowel shortening	common	uncommon
fistulas	no	yes
abscesses	no	yes
rectum involvement	yes	no
carcinoma	2-5%	rare
depth of inflammation	superficial	transmural
reduced Goblet cell	yes	no
vascularity	marked	not prominent
dysplasia	common	uncommon
lymphoid follicles	uncommon	prominent

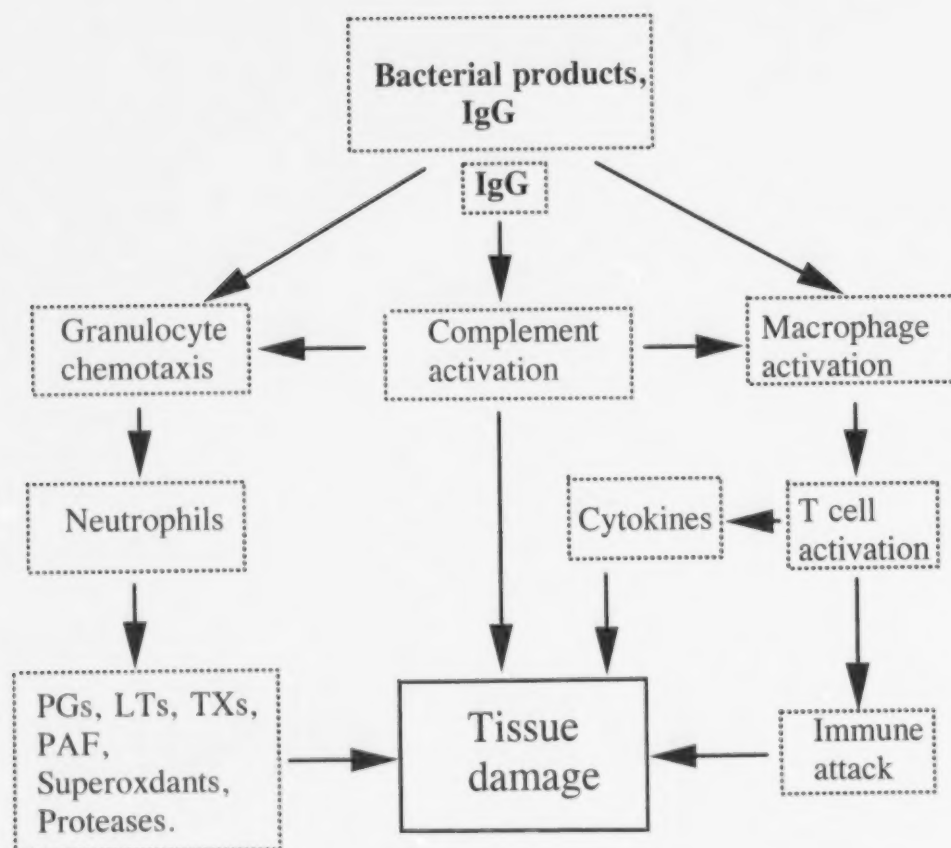


Figure 2-1. Mechanisms of tissue damage in IBD.

CHAPTER 3

GLUCOCORTICOIDS IN IBD: ANTI-INFLAMMATORY PHARMACOLOGY, SIDE-EFFECTS, AND COLON- SPECIFIC DELIVERY

Glucocorticoids are synthesized in the adrenal cortex and then released into the blood stream. The significance of adrenal glands was recognized in 1855 when Addison described the syndrome of hypocorticism (Addison's disease) (1). Cushing described the syndrome of hypercorticism (Cushing's syndrome) in 1932 (2), and these descriptions established the foundations of knowledge about glucocorticoid toxicity. Glucocorticoids were first isolated in 1943 (3), and most artificial glucocorticoids were synthesized from the 1940's to the 1960's.

3.1 CHEMISTRY

The basic chemical structure of glucocorticoids consists of 21 carbon atoms with a total of 4 rings (three 6-carbon rings and one 5-carbon ring). The anti-inflammatory activity of glucocorticoids is determined by the ketone oxygen at C-3, the unsaturated bond between C-4 and C-5, the hydroxyl group at C-11, and the ketone oxygenate C-20 (4). The structures which involve anti-inflammatory activity are shown in Figure 3-1. In order to increase anti-inflammatory potency and decrease side-effects, synthetic analogues have been developed using substitution at sites on the cortisol molecule. As shown in Figure 3-2, methylprednisolone (MP) is made by adding a methyl group at the 6- α position of the prednisone form. Dexamethasone (DX) is made by adding a fluorine atom at the 9- α position (giving enhanced glucocorticoid activity) and a methyl group at the C-16 α position (Figure 3-3). The biological half-life of DX is increased to 36-45 hours. Budesonide (BD) is related to 16- α -hydroxyprednisolone (5). Table 3-2 shows the activity of some of glucocorticoids.

3.2 PHARMACOLOGY

Glucocorticoids are synthesized in and released from the adrenal cortex. In man, over 90% of the plasma cortisol in circulation is bound to plasma proteins. Most of the bound cortisol is associated with corticosteroid-binding globulin or transcortin (6), and a small quantity is associated with albumin. The glucocorticoids used in therapy are, in general, bound less tightly to transcortin than is cortisol (7). Plasma-bound glucocorticoids thus provide a moving reservoir and only free glucocorticoids in circulation are active. Glucocorticoids exert most of their effects through specific intracellular receptors. When free glucocorticoids bind target tissue, the concentration of free glucocorticoids is decreased, and if this decrease causes loss of equilibrium between plasma-bound and free glucocorticoids, then glucocorticoid is released from transcortin (8). The metabolism of glucocorticoids includes reductions of the 4, 5 double bond and of the 3-ketone or the 20-ketone by the action of reductase. In the liver, the reductions which happen at 3 or 4-5 position (A ring) presage hepatic sulfoconjugation or glucuronidations of the steroid at the 3-position. The products of reduction are inactive and water soluble and are excreted by the kidney (9). Another metabolic pathway is the continuous interconversion of cortisol-cortisone by 11- β -hydroxysteroid dehydrogenase (10).

As steroid hormones, glucocorticoids act on steroid receptors which are widely distributed in almost all mammalian tissues. Steroid receptors consist of a single polypeptide chain of the molecular weight 90,000 (11). Glucocorticoid receptors were cloned in 1985 by Hollenberg et al (12). The receptors can be divided into three domains: 1) a carboxyl-terminal steroid-binding domain, 2) a DNA-binding domain and 3) an amino-terminal domain of unknown function (13). The receptors are present in the cytosol of responsive tissues and can bind glucocorticoid in a manner proportional to their intrinsic activities. The non-activated glucocorticoid receptor resides in the cytosol with other highly conserved proteins in the form of a hetero-oligomer (14). This molecular structure is comprised of a receptor, heat-shock proteins, and immunophilin (15). The

binding of the receptor to the heat-shock protein 90 facilitates its interaction with the ligand (16). When the ligand binds, the receptor dissociates from the rest of the hetero-oligomer and translocates into the nucleus. Before or after this translocation, the receptor forms homodimers, through sequences present in the DNA and ligand-binding domains (17). After specific interactions with pore-associated proteins, the hormone-receptor complexes enter the nucleus through the nuclear pores (18), then modulate the transcription rate of the corresponding glucocorticoid-responsive genes (19). The hormone-receptor complexes may interact with a number of nuclear proteins to produce the conditions necessary for effective transcription (20). These proteins can relax the DNA away from the nucleosome and thus make it easier for the polymerase to exert its effects. Glucocorticoid response elements in DNA are able to regulate transcription both positively and negatively (21). By stimulating the interaction of the activated hormone-receptor complex with a heterodimer, glucocorticoids facilitate the binding of heterodimer to the activator protein (AP)-1 site of the genes of several growth factors and cytokines and prevent these genes from transcription thus suppressing the production of these products (22). Glucocorticoids also affect RNA translation, protein synthesis, and secretion, and these effects are exerted through the modulation of the transcription of some unknown proteins that bind RNA and alter its translation and degradation rates (23).

A general model of glucocorticoid action can be described in the following manner: 1) the glucocorticoid enters a responsive cell by passive diffusion through the cellular membrane and binds to intracellular receptors 2) this binding with glucocorticoid leads to conformational changes in receptors, and glucocorticoid-receptor complexes bind to the specific DNA sequences (the glucocorticoid regulatory element in cell nucleus); 3) the glucocorticoid modulates the expression of the gene at the transcription level, and this activity affects the synthesis of mRNA. New proteins are synthesized and exert regulatory effects on cell functions (8).

The discovery of the antiinflammatory actions of glucocorticoids was made in the late 1940's (24). It is now clear that glucocorticoids exert their anti-inflammatory effects

through the following mechanisms:

1) Inhibition of release of phospholipase A_2 : Phospholipase A_2 is present in mammalian plasma membrane. When released from the membrane, phospholipase A_2 cleaves arachidonic acid from glycerophospholipids. Arachidonic acid stimulates the production of prostaglandins and thromboxanes through the cyclo-oxygenase pathway and leukotrienes through the lipo-oxygenase pathway (25). The metabolites of arachidonic acids are known to be inflammatory mediators and to play an important role in IBD (26). Glucocorticoids inhibit the release of phospholipase A_2 from the membrane either directly or by the synthesis of a protein which was isolated and named by three groups of investigators at the same time and has subsequently been renamed as lipocortin (27).

2) Immune suppressive effect: Glucocorticoids can cause lymphocytopenia in humans by inducing redistribution of circulating lymphocytes to the bone marrow (28). In rat and some other species, glucocorticoid causes lymphocytopenia by inducing cell death. T cell proliferation begins when antigens bind to the T cell receptor and initiate a cascade of events leading to the production of interleukin-2 and other cytokines, as well as the induction of high-affinity interleukin-2 receptors. The binding of interleukin-2 to its receptors promotes proliferation of T cells, generation of effector, suppressor, and stimulation of cytotoxic functions. Glucocorticoids suppress the T cell proliferation stimulated by antigens (29) and also suppress the production of IL-2, thereby inhibiting the activation of T cells and their lymphocyte cytotoxic functions (30). Glucocorticoids also inhibit the production of other cytokines (IL-3, IL-4, IL-6, and interferon- γ) essential for T cell function (31, 32). As well, glucocorticoids suppress B cell proliferation (33) and serum IgG function (34). This suppression is the result of an initial increase in immunoglobulin catabolism by a decreased synthesis. The mechanism responsible for the suppression is thought to involve the inhibition of cytokines by glucocorticoids. Because cytokines are involved in immunoglobulin synthesis, the decreased immunoglobulin production might result from decreased accessory or helper T cell activations.

3) Inhibition of neutrophils: Glucocorticoids inhibit the immigration of neutrophils

into the sites of inflammation. They also inhibit the chemotactic response of neutrophils to a variety of chemoattractants (35). It has been demonstrated that glucocorticoids inhibit the release of granule-associated enzymes, and the superoxide anion (O_2^-) by neutrophils during contact with chemotactic peptide N-formyl-methionine-leucine-phenylalanine (FMLP) in a dose-dependent manner (36). The major effect of glucocorticoids on neutrophils is the inhibition of neutrophil adhesion to endothelial cells. This effect diminishes the entrapment of neutrophils in the inflamed site and likely causes a characteristic neutrophilia (37).

4) Inhibition of macrophages: Macrophages play a central role as antigen-presenting accessory cells during the induction of cell-mediated and humoral immune responses (38). During inflammation, the rate of monocyte and macrophage production increases up to 4 times the normal rate (39). Glucocorticoids significantly reduce the number of macrophages in blood and other organs (40) and also reduce the production of IL-1 by macrophages (41). Glucocorticoids inhibit the production of arachidonic acid metabolites (PGs, LTs and TxS), tissue damaging enzymes, and cytokines by macrophages (42,43).

5) Inhibition of mast cells: In man mast cells can be found in lymphoid tissue, thymus mesentery, and the submucosal and subserosal layers of the digestive tract (44). Mast cells release histamine and arachidonic acid metabolites (45). Glucocorticoids inhibits the release of histamine and arachidonic acid metabolites by mast cells (46,47).

6) Stabilization of endothelial cells: The function of endothelial cells affects homeostasis, vascular permeability, and exudation of leukocytes at inflammatory sites. In IBD, endothelial permeability is increased, and endothelial function is damaged. Glucocorticoids inhibit vascular permeability (37). They also inhibit the attachment of endothelial leucocyte adhesion molecules to endothelial cells (48).

3.3 SIDE EFFECTS

Exogenous glucocorticoid therapy is potentially hazardous. It can cause side-

effects in virtually every system in the body. The common side-effects are hyperglycemia, diabetes mellitus, osteoporosis, and Cushing's syndrome.

3.3.1 Hyperglycemia and Diabetes Mellitus

Glucocorticoids cause hyperglycemia through multiple mechanisms. They increase the flow of alanine from skeletal muscle to liver to increase gluconeogenesis (49) and the activity of rate-limiting enzymes for glyconeogenesis (50). Glucocorticoids increase the level of circulating glucagon which accelerates glyconeogenesis in the liver (49) and they also increase tissue resistance to insulin activity (51), so that the insulin's ability to inhibit hepatic glucose output and promote glucose uptake is diminished.

3.3.2 Osteoporosis

Clinical osteoporosis will develop in an estimated 50% of patients undergoing glucocorticoid therapy (52). The mechanisms responsible for this side-effect are based on the fact that glucocorticoids decrease osteoblast recruitment and activity (53, 54), intestinal calcium absorption (55), and renal reabsorption of calcium (56). The results of these changes are hypercalciuria and hyperphosphaturia (57). Glucocorticoids have an inhibitory effect on the growth and replication of cultured osteoblast cell lines (58) and suppress the formation of osteocalcin by human osteoblasts (59). This effect may occur because of an interference with the surface receptor for 1,25-dihydroxy vitamin D. The indirect bone-resorptive effects of glucocorticoids are probably mediated through an impairment of vitamin D-induction of calcium absorption (60).

3.3.3 Cushing's syndrome

In exogenous glucocorticoid-induced Cushing's syndrome, adrenocorticotrophic hormone (ACTH) is suppressed. This suppression happens through two mechanisms. The first one is a fast feedback mechanism which inhibits the release of corticotrophin releasing hormone (CRH) and ACTH but does not inhibit their synthesis (61). Fast feedback is detectable within minutes of the administration of glucocorticoids and lasts while glucocorticoid levels rise. The second mechanism is that of delayed feedback, which occurs after about a 30 minute delay. Delayed feedback has two components, those

of intermediate feedback and slow feedback. Intermediate feedback occurs 2-8 hours after administration of glucocorticoids. It inhibits release of ACTH and CRH, inhibits synthesis of CRH but not ACTH. Slow feedback occurs 10 or more hours after exposure to glucocorticoids. It inhibits the release and synthesis of both ACTH and CRH (61). Delayed feedback involves the typical mechanism of steroid action on the transcription of the genetic message. For example, glucocorticoids inhibit ACTH synthesis by decreasing the synthesis of the mRNA encoding for pro-opiomelanocortin, the precursor of ACTH.

3.4 COLON-SPECIFIC DELIVERY

Since their early use, glucocorticoids have proved to be very powerful anti-inflammatory drugs. Unfortunately, they also caused severe side-effects (as mentioned above), and these side-effects often hindered the use of the glucocorticoids. Different methods designed to increase the efficacy and decrease the side-effects of glucocorticoids have been explored.

3.4.1 Local therapy

In early studies on proctitis and distal colitis, retention enemas or foams containing hydrocortisone, prednisolone and their 21-ester were used as local therapies (62, 63). The dose for local therapy was similar to that used in systemic therapy. It soon became evident, however, that glucocorticoids used locally not only provided local action but also suppressed plasma cortisol levels - an indication of its systemic action (64). Prednisolone metasulphabenzoate has been used to achieve slower absorption of prednisolone, since the conjugate was a larger molecule (65). However, the conjugate produced sulfa-like side-effects (66). Some authors have claimed that hydrocortisone and prednisolone or their esters are not good choices if the medical aim is local therapy of the rectal mucosa (67).

3.4.2 Newer glucocorticoids with high first-pass rates of metabolism

Newer glucocorticoids have also been synthesized and tested clinically. Prednisone and cortisone are inactive and must be activated in the liver (68). When a patient has a

diseased liver, prednisone and cortisone may not be activated. The synthesis of prednisolone and hydrocortisone resolved this problem, because these drugs are active and produce an enhanced anti-inflammatory effect and a reduced Na retention effect. The other side-effects caused by these drugs remain unchanged because the first-pass metabolic rates are low for both drugs (50% for hydrocortisone and 20% for prednisolone [67]). It was thought that in order to improve local therapy of IBD, the glucocorticoids must have a high first-pass metabolism in the portal circulation.

Tixocortol pivalate and budesonide are two typically high first-pass metabolized glucocorticoids. Tixocortol pivalate is a recent derivative of the hydrocortisone structure. The $-\text{COCH}_2\text{OH}$ in the 17- β side chain is transformed into $-\text{COCH}_2\text{SH}$ and esterified via the thiol group (69). Tixocortol pivalate has a high first-pass metabolic rate (80-90%) with the same anti-inflammatory efficacy as hydrocortisone. Indeed, in humans the rectal administration of 500-2000 mg of tixocortol pivalate did not affect plasma cortisol levels (70). However, tixocortol pivalate had a low anti-inflammatory potency and a high plasma clearance (71). The drug's very low local potency excludes its use via sustained release oral formulations.

A newer glucocorticoid with a high first-pass metabolism is budesonide (BD). BD is a 17- α substituted glucocorticoid with 200 times the anti-inflammatory potency of hydrocortisone with a 90% first-pass elimination rate. Being highly water soluble, BD is easily dissolved and absorbed into the intestine after oral or rectal administration. Because of its high first-pass elimination, BD is rapidly metabolized in the liver by oxidative biotransformation into metabolites which have at least 100 times lower glucocorticoid potency than that of the intact compound (72,73). Numerous reports have shown that an enema with 2 mg BD achieves more rapid healing of active distal ulcerative colitis than can be achieved with 31 mg prednisolone and that this healing is not accompanied by apparent systemic side-effects (74).

3.4.3 Colon-specific 5-ASA prodrugs

Research to develop a better agent for oral administration in the treatment of IBD

is important because local therapy administered rectally is only effective for lesions in the distal part of the colon. Consequently, the concept of a colon-specific prodrug delivery system was developed. Prodrugs are pharmacologically inactive compounds resulting from chemical modification of a biologically active compound (75). Prodrugs have different physicochemical properties than the active, free molecule and release the active compound in vivo in response to enzymatic or hydrolytic cleavage. In this manner, the availability of the active compound is controlled. Prodrugs designed for localized colonic activity should not be absorbed from the other parts of the GI tract after oral administration and the rate of conversion to the active drug should be faster in the colon than in the small intestine.

The first colon-specific anti-inflammatory drug was sulfasalazine which was introduced for rheumatoid arthritis in the early 1940s (76). Amazingly, it took more than 30 years for researchers to realize that sulfasalazine is a prodrug. The active agent in this prodrug is 5-aminosalicylic acid (5-ASA) and the inactive part is sulfapyridine (77). These two entities are connected through an azo bond. In the colon, bacterial enzymes break the azo bond and release 5-ASA which then exerts its anti-inflammatory effect locally, and is metabolised into inactive *N*-acetyl-5ASA by bacteria in the intestinal lumen, intestinal mucosal cells and hepatocytes (78,79,80). *N*-acetyl-5ASA is eliminated in the urine via the kidney (81). Another 5-ASA prodrug, olsalazine, is synthesized with two 5-ASA components which are connected by an azo bond.

5-ASA inhibits both cyclo-oxygenase and lipo-oxygenase pathways; therefore, it inhibits prostaglandin (PG), thromboxane (TX), and leukotriene (LT) synthesis and release (82). 5-ASA is also an inhibitor of platelet-activating factor (PAF), cytokines, mast cells, and basophil-stimulated histamine release. It can also scavenge free oxygen radicals (83). Table 3-1 shows some of 5-ASA prodrugs in IBD treatment.

5-ASA prodrug side-effects include male infertility, headache, nausea, epigastric distress, and diarrhea (84). Due to their relatively low anti-inflammatory potency, 5-ASA prodrugs are only used for mild and moderate cases of IBD.

3.4.4 Colon-specific glucocorticoid prodrugs

Another group of prodrug candidates for colon-specific delivery are the glucocorticoids. Volker described the first colon-specific delivered dextran-prodrug delivery system in 1962 (85). Dextran conjugated with a dye delayed urinary excretion of the dye after oral administration. Larsen et al demonstrated that a colon-specific prodrug, an esterified naproxen-dextran conjugate, was initially degraded by the action of bacterial dextranases into smaller fragments and then degraded further by esterases prior to the release of the active drug into the colon (86-89). The prodrug remained intact and unabsorbed from the small intestine, whereas free naproxen was completely released in the colon. Thus, dextran was shown to have ideal characteristics for colon-specific prodrug. Friend and colleagues were the first to work on colon-specific glucocorticoid delivery systems (90, 91). Figure 3-4 illustrates the principle of glucocorticoid prodrug delivery. The carriers that have been used in the synthesis of glucocorticoids include dextran, β -D-glucuronide, and β -D-glycoside. The structures of dextran prodrugs and β -D-glucuronide prodrugs are depicted in Figure 3-5. The glucocorticoid-dextran prodrug conjugates have different chemical stabilities. For instance, at 37°C and pH 6.8, the degradation half-life is 75 hours for dexamethasone-succinate-dextran (DS-dextran), 103 hours for dexamethasone-glutarate-dextran (DG-dextran), and 82 hours for methylprednisolone-succinate-dextran (MPS-dextran) (93). These differences suggest that prodrugs having longer hydrocarbon chains are more stable than those with shorter hydrocarbon chains. Incubation of DX-21-hemiester with rat GI contents showed that the hemiester is hydrolyzed throughout the GI tract but the greatest esterase activity occurred in the small intestine. Dextran prodrugs resist being broken down in the small intestine since dextran sterically hinders enzymatic hydrolysis of the ester bonds (92). However, once dextran is split from the prodrug, esterases produced by colonic bacteria further degrade the ester bond and the glucocorticoid is released into the colonic lumen (92). The release of the glucocorticoid was demonstrated to be low in the small intestine by McLeod et al and substantially higher in the large intestine (92). Consequently, the concentration

of the free drug in systemic circulation was very low whereas relatively high concentrations were found in the colonic contents/mucosa after administration of the prodrugs. When incubated with caecal contents at different pH's ranged from 4.3 to 7.9, all the prodrugs showed a maximal hydrolysis of corticosteroid-hemiesters at pH 6.9, indicating that the enzyme in the caecum was an alkaline esterase (91). It was also found that anaerobic condition did affect the hydrolysis of DS-dextran in ceacal contents (91). Perhaps the most significant finding about these prodrugs was that the concentrations of free drug released from the prodrugs were almost identical throughout the entire large intestine (93). These prodrugs, therefore, have the potential to be clinically effective for both proximal and distal colitis.

Friend and Chang proposed a glucocorticoid-glucoside prodrug (94) and tested it in rats and guinea pigs (95,96). They found that although a measurable amount of dexamethasone was delivered to the guinea pig caecum and colon, the efficiency of delivery was lower than expected. The possible reasons involved premature hydrolysis due to the activity of bacterial or host β -D-glucosidase, and direct absorption of the prodrug from the lumen of the GI tract. More recently, Friend et al synthesized two prodrugs using β -D-glucuronide, including dexamethasone- β -D-glucuronide (DX-glrd) and budesonide- β -D-glucuronide (BD-glrd) (Figure 3-5) and conducted a GI tract stability study on DX-glrd (97). Using conventional, colitic, and germ-free rats, they found that the prodrug was broken down when incubated with the intestinal contents of the caecum and colon of conventional and colitic rats. It was also confirmed that β -D-glucuronidase activity was mainly associated with colonic bacteria; enzyme activity was low in the luminal contents and in the mucosa of the small intestine in all three groups. Enzyme activity was low in the luminal contents and mucosa of the whole GI tract in germ-free animals.

The details of the synthesis of these glucocorticoid prodrugs have been described by Friend et al (90, 91, 97). Table 3-3 summarizes the composition and release site of the prodrugs tested.

Budesonide has also been made into a coated-capsule preparation which allows for controlled release in the distal small intestine and proximal colon. In a double-blind, multi-center trial, this preparation was found to be very effective, inducing remission in patients with mild to moderately active Crohn's disease at a daily dose of 9 mg (98). However, this dose also caused impaired adrenal function in 50% of the patients after 8 weeks.

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Table 3-1. Oral 5-ASA preparations

Preparation	Formulation	Delivery
<i>Azo-bond</i>		
Sulfasalazine	Sulfapyridine-carrier	Colon
Olsalazine	5-ASA dimer	Colon
<i>Delayed-release</i>		
Mesasal [®]	Eudragit L	Distal ileum, colon
Salofalk [®]		
ASACOL [®]		
<i>Sustained-release</i>		
Pentasa [®]	Ethylcellulose microgranules	duodenum-colon

Table 3-2. Potencies of corticosteroids

Corticosteroid	GC ^a activity	MC ^b activity	Half-life in tissue(h)	Equivalent ^c dose(mg)
Cortisol	1	1	8-12	20
Cortisone	0.8	0.8	8-12	25
Prednisone	4	0.8	12-36	5
Prednisolone	4	0.8	12-36	5
6 α -Methylprednisolone	5	0.5	12-36	4
Dexamethasone	25	0	36-54	0.75
Budesonide	200	0	36-54	0.1

^a glucocorticoid.^b mineralocorticoid.^c For oral administration.

Table 3-3. Synthesized glucocorticoid prodrugs

Prodrugs	Carrier	Release site
<i>Dextran prodrugs</i>		
Dexamethasone-succinate-dextran	dextran	caecum-colon
Dexamethasone-glutarate-dextran	dextran	caecum-colon
Methylprednisolone-succinate-dextran	dextran	caecum-colon
<i>Glucuronide prodrugs</i>		
Budesonide- β -D-glucuronide	β -D-glucuronide	caecum-colon
Dexamethasone- β -D-glucuronide	β -D-glucuronide	caecum-colon

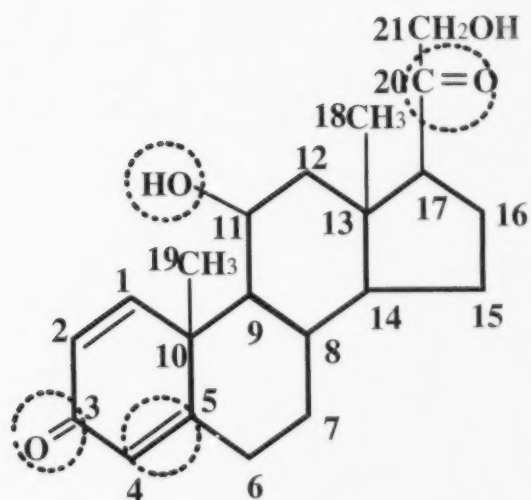


Figure 3-1. Basic structure of 21-C steroid and the structures with anti-inflammatory activity.

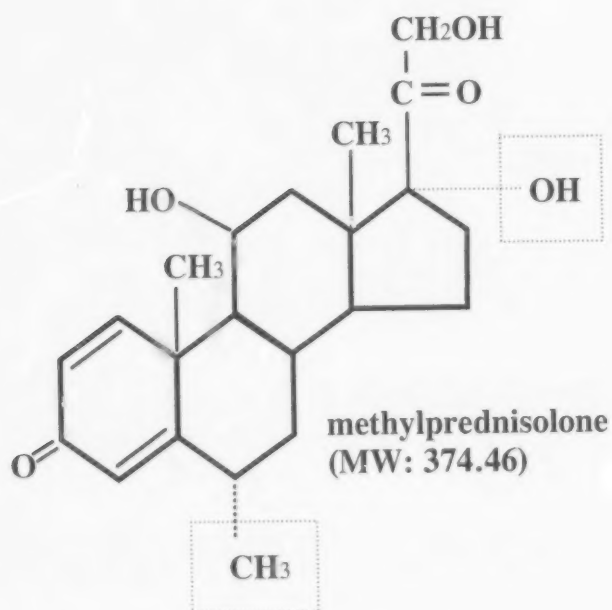
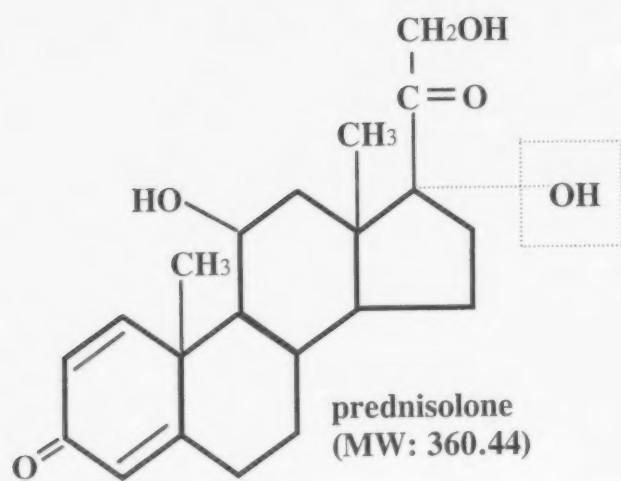


Figure 3-2. Structures of prednisolone and methylprednisolone.

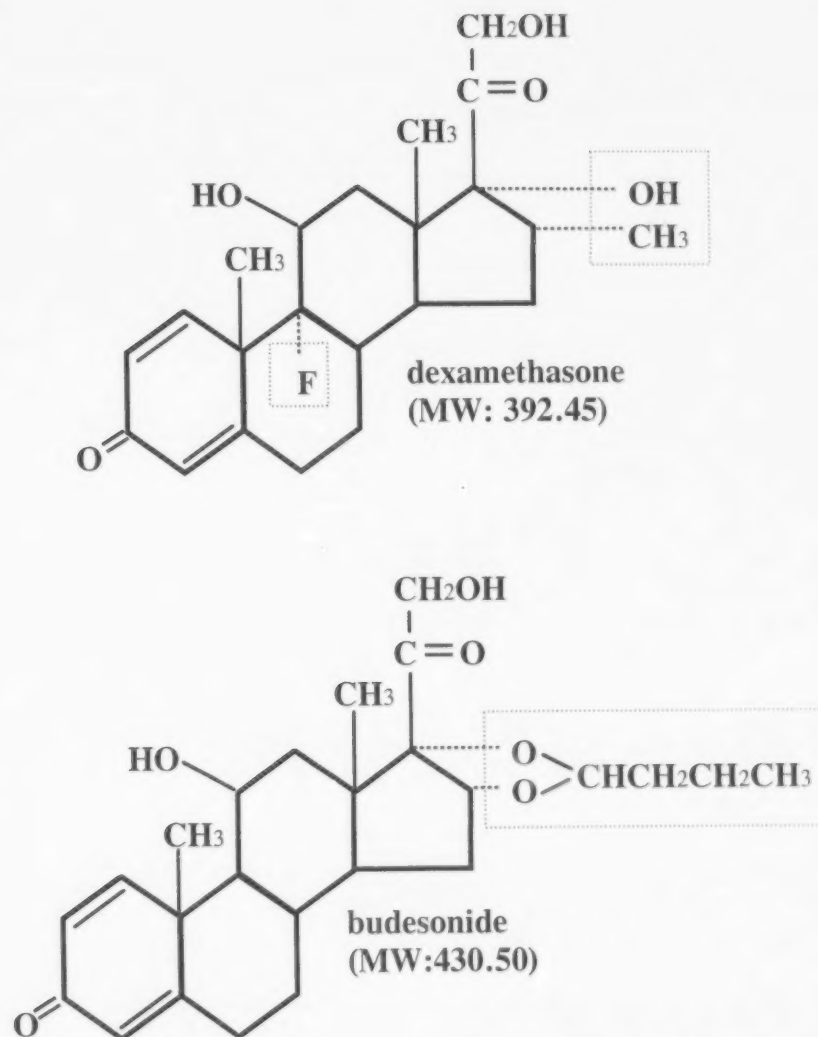


Figure 3-3. Structures of dexamethasone and budesonide.

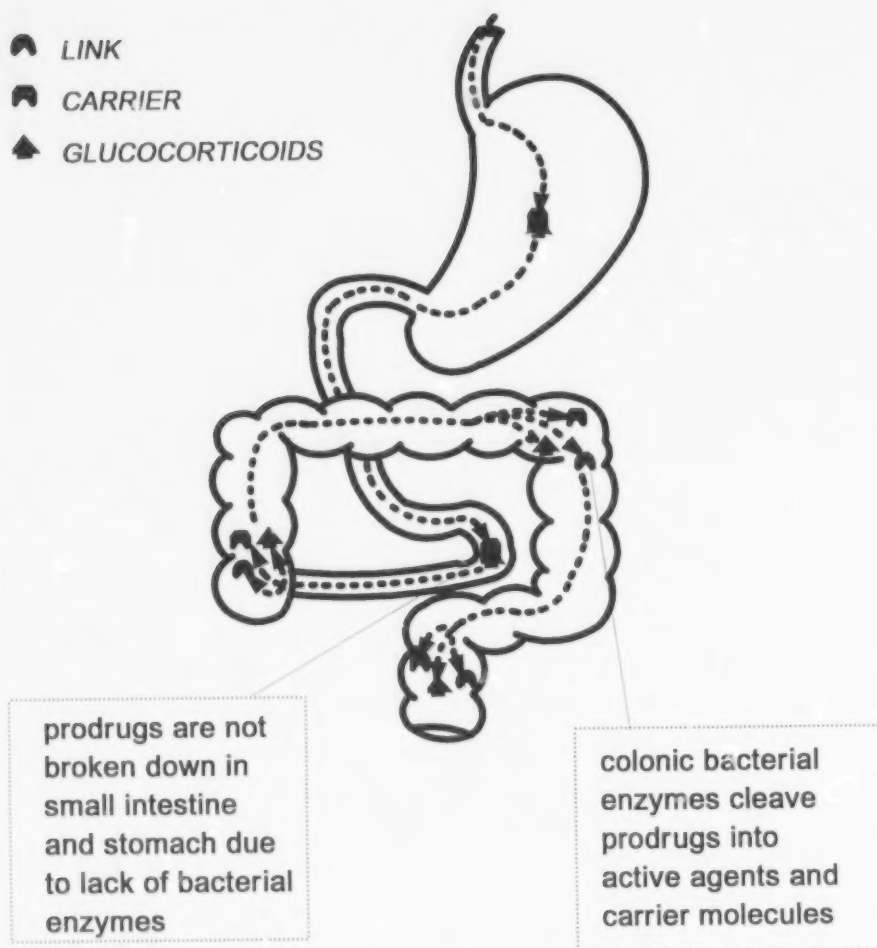
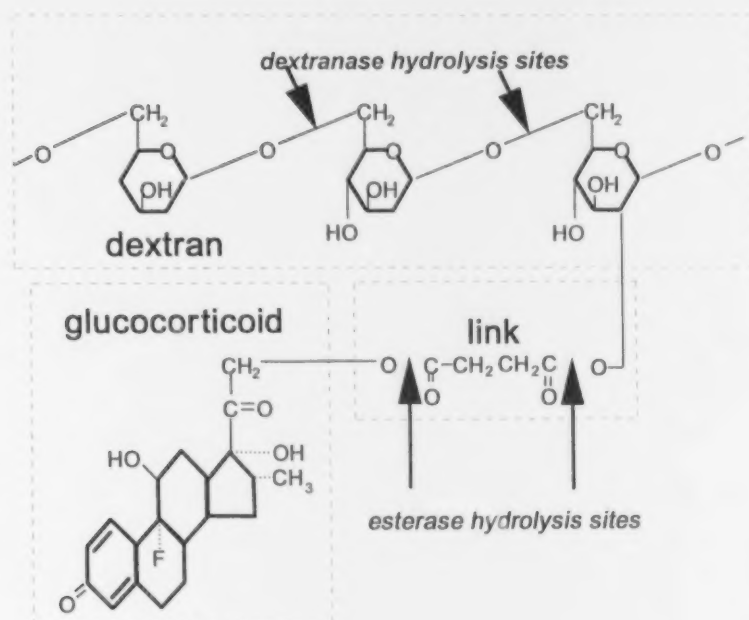


Figure 3-4. Glucocorticoid prodrug delivery and release.

Glucocorticoid-Dextran Prodrugs



Glucocorticoid-beta-D-Glucuronide Prodrugs

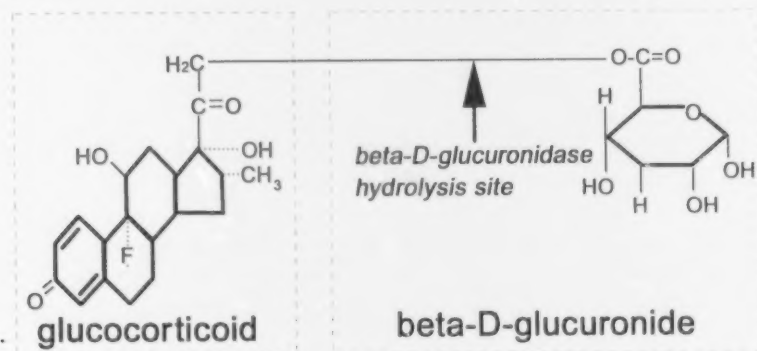


Figure 3-5. Different glucocorticoid prodrugs.

CHAPTER 4*

EFFICACY STUDIES

4.1 INTRODUCTION

In this study the order of anti-inflammatory potency of glucocorticoids is: budesonide (BD) > dexamethasone (DX) > methylprednisolone (MP) (1). The release of free drugs from their prodrugs depends on intestinal bacterial enzyme activities and the structures of the carriers in the prodrugs. As before, the anti-inflammatory efficacy of the free drugs depends on their structures. A number of studies have shown that glucocorticoid prodrugs release active agents in the cecum and colon only and lead to high colonic glucocorticoid concentration (2,3). In this study, glucocorticoid- β -D-glucuronide prodrugs and glucocorticoid dextran prodrugs were examined. In dextran-prodrugs succinate has a hydrocarbon chain with 4 carbon atoms and glutarate has a carbon chain with 5 carbon atoms. It has previously been demonstrated that dextran prodrugs with a longer hemiester hydrocarbon chain are more stable than those with a short hydrocarbon chain (4). Therefore, the anti-inflammatory efficacies of different glucocorticoids delivered by the same carrier are compared as well as the anti-inflammatory effects of the same glucocorticoid delivered by different carriers.

4.2 MATERIALS AND METHODS

DX, MP, BD, and their prodrugs, dexamethasone-succinate-dextran (DS-dextran), dexamethasone-glutarate-dextran (DG-dextran), methylprednisolone-succinate-dextran (MPS-dextran), budesonide- β -D-glucuronide (BD-glrd), and dexamethasone- β -D-glucuronide (DX-glrd) were synthesized by Dr. David Friend and Dr. Thomas Tozer at

*Portions of Chapter 4 and Chapter 5 have been published. 1. N Cui, DR Friend, RN Fedorak: A budesonide prodrug accelerates treatment of colitis in rats. *Gut* 1994; 35: 1439-1446; 2. McLeod AD, Fedorak RN, Friend DR, Tozer TN, and Cui N: A glucocorticoid prodrug facilitates normal mucosal function in rat colitis without adrenal suppression. *Gastroenterology* 1994; 106: 405-413

Stanford Research Institute, Menlo Park, California, USA (Table 3-3).

4.2.1 Animals

Male Sprague Dawley rats (250-275 g, Biotron, University of Alberta, Edmonton, AB, Canada) were provided water and laboratory chow at libitum. The rats were kept on a 12-h light/12-h dark cycle. There were 6 rats in each experimental group.

4.2.2 Chemicals

Glacial acetic acid was from Fisher Scientific (Nepon, ON, Canada). All remaining reagent grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

4.2.3 Calculation of doses:

Doses of DX[Molecular weight (MW): 392.45], DX-glrd (MW: 626.00), MP (MW: 374.46), BD (MW: 430.50), and BD-glrd (MW: 606.60) were calculated according to their molecular weights.

$$\text{Therefore, Dose}_w = \text{Dose}_D \times \text{MW} \times 0.25 \text{ kg}$$

Where: Dose_w = weights of free corticosteroids or prodrugs (μg) for one rat per day

$$\text{Dose}_D = \text{designed doses } (\mu\text{mol/kg/d})$$

MW = molecular weights of free corticosteroids or prodrugs

Doses of DS-dextran [The degree of substitution (D.S): 8.6], DG-dextran (D.S: 5.7), and MPS-dextran (D.S: 10.5) were calculated according to their D.S. The degree of substitution is the amount of corticosteroid (mg) in 100 mg dextran prodrug.

$$\text{Therefore, Dose}_w = \text{Dose}_D \times (\text{MW} \times 100/\text{D.S}) \times 0.25 \text{ kg}$$

Where: Dose_w = weights of prodrugs (μg) for one rat per day

$$\text{Dose}_D = \text{designed doses } (\mu\text{mol/kg/d})$$

MW = molecular weights of free corticosteroids

D.S = the degree of substitution of corticosteroid

4.2.4 Induction of colitis

The induction of colitis is illustrated in Figure 4-1. Non-fasting male Sprague-

Dawley rats were anaesthetized via intraperitoneal injection of pentobarbital (55 mg/kg) and atropine (0.5 mg/kg). The colon was isolated by way of a sterile midline abdominal incision, and the junction of the caecum and ascending colon was occluded with a reversible ligature; care was taken to avoid compromising neural or vascular integrity. The colon was cleansed of its luminal contents with 154 mM sodium chloride solution at 37°C, and the residual fluid was manually expressed through the rectum. Two mL of 4% Acetic acid (AAC) was injected into the lumen of the colon through a 26 gauge needle passed obliquely through the colonic wall just distal to the occluding ligature. Immediately thereafter, 10 mL of air was injected to clear the acetic acid from the colon. The occluding ligature was removed and the midline incision closed. The animals were allowed to recover from the anaesthesia in a light-cycled room that provided free access to standard rat chow pellets (5001, Purina Mills Inc., St. Louis, MO, USA) and water. All in vivo studies outlined below were carried out 72 hours after induction of colitis.

4.2.5 Administration of Drugs

DX and its prodrugs, DX-glrd, DG-dextran, DS-dextran, MP and its prodrug MPS-dextran, BD and its prodrug BD-glrd, were administered by oral gavage 24 and 48 hours after induction of colitis. The prodrug conjugate solutions were prepared immediately before their oral administration by mixing the appropriate volume of stock solution with 154 mM sodium chloride to a total volume of 1 mL (pH 7.4). Rats were administered doses of free drugs or their prodrugs by oral gavage, ranging from 0.0137 to 0.44 $\mu\text{mol/kg/d}$.

4.2.6 In Vivo Colonic Fluid Absorption

In vivo net colonic fluid absorption was used as one measure to assess repair of mucosal integrity during experimentally-induced colitis and it was assessed as described previously (5). Seventy-two hours after induction of colitis rats were anaesthetized with pentobarbital (55 mg/kg) and atropine (0.5 mg/kg) and maintained at 37°C using a thermostatic heat lamp. The intestinal tract was exposed through a midline abdominal incision. An occluding ligature was placed at the caecal-ascending colon junction.

Sodium chloride solution (154 mM, 37°C) was instilled into the proximal colon via a cannula inserted through an incision just distal to the proximal occluding ligature to flush out the luminal contents of the colon. Residual saline was emptied manually. A 12-cm long intestinal loop, beginning 2 cm below the caecal-colonic junction and extending distally to the peritoneal reflection, was created with ligatures. In isolating the loop, care was taken not to compromise mesenteric, vascular or neural integrity. A 27 gauge needle was inserted obliquely through the outer muscle layer along the antimesenteric border, and 2 mL of sodium chloride solution (37°C, 154 mM) was instilled into the empty loop. In all cases, no fluid leakage was detected, and the loop was only mildly distended. The viscera were returned to the abdominal cavity and the incision was closed. Sixty minutes after abdominal closure, the animals were given a pentobarbital overdose (240 mg/kg) and the intestinal loops were removed. The length of each loop was recorded. The intestinal loops were weighed, both full and empty, to determine residual intraluminal volume. Results were expressed as the difference between initial and residual loop volumes over the hour per centimetre of intestine.

The nonlinear regression program *Minim* (Version 1.8a, Dr. R. Purves, Department of Pharmacology, University of Otago, Dunedin, New Zealand) was used to fit the Hill equation to the colonic water-absorption data and for ED₅₀ calculation. To incorporate both positive and negative values, the equation was modified to include the term, Effect₀ (μL/hr/cm), as follows:

$$\text{Effect} = \text{Effect}_0 + (\text{Effect}_{\max} \cdot \text{Dose}^\gamma) / [(\text{ED}_{50})^\gamma + \text{Dose}^\gamma]$$

where:

Effect = Water flux, μL/hr/per cm of intestine

Effect₀ = Water flux in animals with acetic acid-induced colitis, μL/hr/per cm of intestine

Effect_{max} = -Effect₀ + Control (Control is the value in animals not treated with either 4% acetic acid or drug), μL/hr/per cm of intestine

γ = Hill coefficient

ED_{50} = Daily dose which gives half-maximal effect ($\mu\text{mol/kg/day}$)

4.2.7 Colonic Macroscopic Ulceration

The colon was rapidly excised, opened along its mesenteric border, and gently rinsed of its luminal contents with an iced 154 mM solution of sodium chloride. The colon was then placed flat, mucosal surface upwards, on a glass plate chilled to 4°C. A transparent acetate was placed 5 mm above the mucosal surface and the area of ulceration and total surface area were traced by a single observer (Cui N). Areas in square centimetres were then calculated using a Zeiss computerized videoscope (Videoplan, Carl Zeiss Co., Toronto, ON, Canada). The data were also transformed into a term expressing the improvement in macroscopic ulceration, in which control animals scored 0% and those treated with acetic acid but no drug scored 100%.

4.2.8 Myeloperoxidase (MPO) Activity

Accumulation of neutrophils in inflamed tissue is one of the characteristics in active IBD and the products of neutrophils cause tissue injury (6). An assay of colonic myeloperoxidase activity was used to quantify neutrophil infiltration. The entire length of colon, from the ascending-caecal junction to rectum, was homogenized with a polytron homogenizer (Brinkman Instruments, Rexdale, ON, Canada) three times for 30 seconds each at 4°C in 5 mL of 0.5 % hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6). The homogenate was then sonicated for 10 seconds and centrifuged. Supernatant (0.1 mL) was combined with 2.9 mL of 50 mM phosphate buffer (pH 6) containing 1.05 mM *o*-anisidine hydrochloride and 0.15 mM hydrogen peroxide (7). Changes in absorbance at 460 nm were measured with a Beckman DU-6 spectrophotometer (Beckman Instruments Inc., Irvine, CA, USA). One unit of myeloperoxidase activity was defined as that degrading 1 μM of peroxide per minute at 25°C.

4.2.9 Statistics

Data are expressed as mean $\pm$ SD. Statistical analysis of the data was performed by repeated-measure analysis of variance. When the overall analysis showed significance,

the Students t-test was used to examine the location and significance of differences.

4.3 RESULTS

4.3.1 In Vivo Colonic Fluid Absorption

Net colonic fluid absorption in controls was unaffected by dosing with the highest dose ($0.44 \mu\text{mol/kg/d}$) of DX, DX-glrd (Figure 4-2).

Upon induction of colitis with 4% acetic acid, colonic fluid absorption became negative, ie, there was net secretion of fluid into the colonic lumen ($p < 0.001$ compared with sham operated control), suggesting the colonic absorptive function was impaired.

4.3.1a Effect of BD and BD-glrd

The colonic fluid absorption data in colitic animals for free BD and its conjugate BD-glrd are shown in Figure 4-3. Treatment with BD-glrd significantly improved in vivo fluid absorption relative to free BD at each dose tested. Fluid absorption data was then fitted to the modified Hill equation to calculate the ED_{50} (effective daily dose which gives half-maximal effect) for BD. The ED_{50} ($\pm$ SD) for BD-glrd was $0.0132 \pm 0.0034 \mu\text{mol/kg/d}$ and for BD $0.0514 \pm 0.0128 \mu\text{mol/kg/d}$ (Table 4-1). The 4-fold difference between these two values is highly significant ($p < 0.001$).

4.3.1b Effect of DX, MP, and corresponding prodrugs

The colonic fluid absorption data in colitic animals for free DX, DS-dextran, DG-dextran, free MP, and MPS-dextran are shown in Figure 4-4 and Figure 4-5. Treatment of colitis animals with DS-dextran, DG-dextran, or MPS-dextran significantly accelerated the restoration of normal fluid absorption relative to treatment with free glucocorticoids. The ED_{50} values for the compounds effect on colonic fluid absorption are summarized in Table 4-1. Comparing ED_{50} values, DS-dextran was 9 times more potent and DG-dextran was 3 times more potent than free DX ($p < 0.001$), and MPS-dextran was 4 times more potent than free MP ($p < 0.001$) (Table 4-1).

4.3.1c Effect of DX and its DX-glrd

As shown in Figure 4-6, both free DX and Dxglrd improved the colonic fluid

absorption. However, the prodrug demonstrated a better effect especially in lower doses. At the lowest dose, the free drug did not have any benefit but the prodrug reversed the net fluid secretion caused by colitis to net fluid absorption. The ED_{50} for DX was $0.180 \pm 0.008 \mu\text{mol/kg/d}$, and for DX-glrd it was $0.0130 \pm 0.008 \mu\text{mol/kg/d}$, showing that DX-glrd was 14 times more potent (Table 4-1). At the highest dose the prodrug increased the fluid absorption to normal.

4.3.2 Colonic Macroscopic Ulceration

Figure 4-7, Figure 4-8, Figure 4-9 and Figure 4-10 show that control animals did not have macroscopic ulceration of the colon but induction of colitis with 4% acetic acid produced gross macroscopic ulcers which were more than 6% of the total colonic surface area ($p < 0.01$ relative to control).

4.3.2a Effect of BD and BD-glrd

Figure 4-7 shows the degree of colonic macroscopic ulceration in each treatment group. Analogous to the results seen with measurement of *in vivo* colonic fluid absorption, treatment with BD-glrd (0.0137 and $0.11 \mu\text{mol/kg/d}$) significantly improved macroscopic colonic ulceration relative to free BD. At the dose of $0.44 \mu\text{mol/kg/d}$, both BD and BD-glrd reduced the ulcer area to a minimum.

4.3.2b Effect of DX, MP, and their dextran prodrugs

Treatment with the DX-dextran prodrug conjugates, DS-dextran and DG-dextran, facilitated repair and significantly improved gross macroscopic colonic ulceration compared to free DX (Figure 4-8). Similar statistically significant results were seen with MPS-dextran compared to free MP (Figure 4-9). Fitting the Hill equation to these data yielded ED_{50} values shown in Table 4-1. ED_{50} values determined from gross colonic ulceration, expressed as a ratio of free glucocorticoid-dextran prodrug conjugate, were again similar to those determined from *in vivo* colonic fluid absorption. DS-dextran was 10 times more potent and DG-dextran 4 times more potent than free dexamethasone ($p < 0.001$). MPS-dextran was 6 times more potent than free MP ($p < 0.001$).

4.3.2c Effect of DX and DX-glrd

As shown in Figure 4-10, both free DX and DX-glrd reduced the tissue damage in a dose-dependent manner. At the highest dose the ulcer area was reduced to a minimum. However, there was no significant difference between the effects of free drug and prodrug.

4.3.3 MPO activity

Figure 4-11, 4-12, 4-13, and 4-14 show that induction of colitis with acetic acid significantly elevated the degree of MPO activity relative to sham operated control ($p < 0.001$).

4.3.3a Effect of BD and BD-glrd

Increasing doses of BD or BD-glrd resulted in reductions in myeloperoxidase activity to normal levels at the highest doses administered (Figure 4-11). However, in contrast to the results seen with colonic fluid absorption and gross macroscopic ulceration measurements, there was no significant difference between BD-glrd and free BD.

4.3.3b Effect of DX, MP, and their dextran prodrugs

As seen in Figure 4-12 and Figure 4-13, increasing doses of free DX and free MP or their respective dextran conjugates resulted in dose dependent reductions in MPO activity to near normal levels at the highest doses administered. There was no difference among the dextran conjugates and their respective free glucocorticoids in the improvement of MPO activity.

4.3.3c Effect of DX and DX-glrd

As shown in Figure 4-14, the MPO activity is decreased in both free drug and prodrug treated groups in a dose dependent manner. At the highest dose the MPO activity was in normal range in prodrug treated animals and the effect was significantly different from that in free drug treated animals.

4.4 DISCUSSION

As seen in our results, at the same doses prodrugs had equal or higher efficacy on

improvement of various parameters in acetic acid-induced rat colitis.

Absorption of water and electrolytes is one of the functions of the colon. In the human, about 500 ml fluid enters the colon from small intestine daily and 350 ml is absorbed (8). Diarrhea occurs when water in the feces is over 200 ml per day (9). In IBD, the absorption of fluid and electrolytes is decreased and in 66-92% of the patients it is even reversed to secretion (diarrhea) (10) due to decreased absorption area by tissue damage, increased intestinal permeability and osmolarity, and secretive stimulation from inflammatory mediators and cytokines (11, 12). Clinically, the frequency and severity of diarrhea is correlated with disease activity, therefore symptoms of diarrhea are used for assessment of the clinical course and response to treatment (10). It has been shown that the extent of *in vivo* colonic fluid absorption is a sensitive measure of functional mucosal absorptive injury in animal IBD model (13).

It has been known that glucocorticoids can increase fluid and electrolyte transport in the intestine (14). The results of the present study showed that free DX and DX-glrd did not significantly increase fluid absorption in normal rats (Figure 4-2), suggest that corticosteroids not only enhance existing intestinal fluid and electrolyte transport, but also facilitate repair of intestinal mucosa and functional fluid absorptive injury, block the effect of inflammatory mediators, and inhibit the migration of neutrophils (15, 16).

These results also showed that all of the prodrugs restored net colonic fluid absorption to a significant degree relative to their parent drugs. The ED_{50} data can be used to compare the relative potency of the prodrugs and free drugs. BD-glrd showed 4-fold increase in relative potency compared to free BD. Dextran prodrugs of DX increased the relative potency 3-fold (DG-dextran) and 9-fold (DS-dextran), relative to free DX. MPS-dextran increased the relative potency 4-fold compared with free MP. DX-glrd increased the relative potency about 16-fold compared with free DX. Among the three dextran prodrugs DS-dextran was the most potent prodrug conjugate in facilitating repair of colitis, as measured by net colonic fluid absorption, with absorption returning to control values at a dose of $0.44 \mu\text{mol/kg/d}$ and was statistically improved relative to free

DX at a dose 100-fold lower (Figure 4-4). MPS-dextran ($0.44 \mu\text{mol/kg/d}$) also returned net colonic fluid absorption to control values (Figure 4-5) whereas DG-dextran ($0.44 \mu\text{mol/kg/d}$) did not (Figure 4-4). Nevertheless, DG-dextran improved colitis and reversed net colonic secretion to net absorption at a significantly lower dose ($0.0034 \mu\text{mol/kg/d}$) than MPS-dextran ($0.055 \mu\text{mol/kg/d}$, Figure 4-4 and Figure 4-5). These results are likely related to the difference between the two carriers and difference of activities between the two free drugs. Succinate has a shorter hydrocarbon chain and is more labile to enzymatic hydrolysis than gutarate. Therefore, the free drug is more readily released, whereas DX is more potent than DG-dextran in terms of antiinflammatory activity. The efficacy of a corticosteroid prodrug is determined by the combined properties of the free drug and the carrier.

Comparing ED_{50} values in Table 4-1, free DX was 3 times more potent than free MP in facilitating healing of colitis as determined by improvement in the *in vivo* net colonic fluid absorption. These results are similar to human studies where DX has been shown to have 5-fold more potent anti-inflammatory properties than MP. Therefore, it also indicates that the efficacy of the DX-dextran prodrugs may be more related to its enhanced anti-inflammatory potency of DX relative to MP.

The ED_{50} of free DX was calculated to be $0.180 \pm 0.008 \mu\text{mol/kg/d}$ while that of free BD was $0.0514 \pm 0.013 \mu\text{mol/kg/d}$. However, the ED_{50} of BD-glrd determined in this study ($0.0132 \pm 0.003 \mu\text{mol/kg/d}$) was similar to that of DX-glrd ($0.0130 \pm 0.008 \mu\text{mol/kg/d}$). The reason that the prodrug of the more potent BD was not more efficacious than DX prodrug with the same carrier is unclear.

The anti-inflammatory effect of these conjugates likely occurred because of higher intraluminal colonic levels of free glucocorticoids being available. Because the prodrugs are poorly absorbed, the conjugates reach the large intestine where they are hydrolysed to free drugs and their carrier molecules. The luminal concentration of free DX in colitic rats treated with DX-glrd was much higher than that in colitic rats treated with free drug (see Chapter 5). The same conclusion was drawn from a comparison of luminal

concentrations of free DX after administration of DX to its dextran prodrugs (3). The locally-attained high concentrations of the free DX is due to local high bacterial hydrolytic activity, which leads to elevated mucosal levels of the free drug. Because of this local delivery effect, lower doses of the conjugates can be used to attain therapeutic levels in the tissues.

Fitting the Hill equation to macroscopic ulceration data for the prodrugs yielded results which were very close to those obtained with the colonic fluid absorption data (Table 4-1, Figure 4-8, and Figure 4-9). The dextran-conjugates were four to ten times more potent than their free glucocorticoids in facilitating the repair of colonic macroscopic ulceration, with DS-dextran more potent than the other conjugates. In contrast to fluid absorption measurements, free MP and DX had a similar potency in healing colonic macroscopic ulceration (ED_{50} : 0.046 ± 0.017 vs 0.049 ± 0.021 μ mole/kg/d, respectively). The improvement of the macroscopic observations by DX-glrd was not consistent with fluid absorption improvement. The reason for this difference between fluid absorption and colonic ulceration remains to be determined. However, previous studies have shown that healing of experimental colitis can occur at differential rates along the crypt-villus axis, with intestinal transport properties being the last to recover from injury (13)

MPO reflects the quantity of neutrophils in the tissue and, subsequently, inflammation (7). While the BD, DX, and MP compounds all reduced MPO activity to near normal levels at the highest doses administered, there was no difference among all the prodrugs and their free glucocorticoids. It has been found that glucocorticoids can decrease the enzymes produced by neutrophils and stabilize lysosomal membrane inside neutrophils (17). In the present study every treatment started 24 hours after the induction of colitis. During this period of time, significant amount of neutrophils had accumulated in the colon already because neutrophils are the cells which first appear in an inflamed area. Thus, it is unlikely that in the inflammatory process the beneficial effect of the prodrugs in facilitating repair of colitis mucosa occurs as a consequence of neutrophil

migration inhibition, more likely it occurs through stabilization of lysosomal membrane. Similar discordant results between intestinal repair and MPO activity (inflammation) during colitis treated with glucocorticoids have recently been described (18).

The efficacy studies demonstrate that the glucocorticoid prodrugs facilitate the return of normal colonic function in rat colitis, at lower doses, relative to their respective free glucocorticoids. Generally, the potency of a prodrug is determined by its intrinsic activity. When hydrolytic activities of luminal bacterial enzymes are the same, its potency is influenced by the specific structures of the carrier.

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Table 4-1. ED₅₀ for improvement of colonic fluid absorption and ulcer healing.

Compound	Colonic fluid absorption ED ₅₀ (μ mol/kg/d) ^a	Colonic ulceration ED ₅₀ (μ mol/kg/d) ^a
Dexamethasone	0.180 $\pm$ 0.0084	0.049 $\pm$ 0.021
Dexamethasone-glutarate-dextran	0.060 $\pm$ 0.0015*	0.011 $\pm$ 0.002*
Dexamethasone-succinate-dextran	0.021 $\pm$ 0.005*	0.005 $\pm$ 0.003*
Methylprednisolone	0.603 $\pm$ 0.227	0.046 $\pm$ 0.017
Methylprednisolone-succinate-dextran	0.136 $\pm$ 0.023*	0.005 $\pm$ 0.004*
Budesonide	0.051 $\pm$ 0.013	—
Budesonide- β -D-glucuronide	0.0132 $\pm$ 0.003*	—
Dexamethasone- β -D-glucuronide	0.0130 $\pm$ 0.008*	—

^aValues represent mean $\pm$ SD of the computer fit of ED₅₀.

*p<0.01 compared with respective free glucocorticoid.

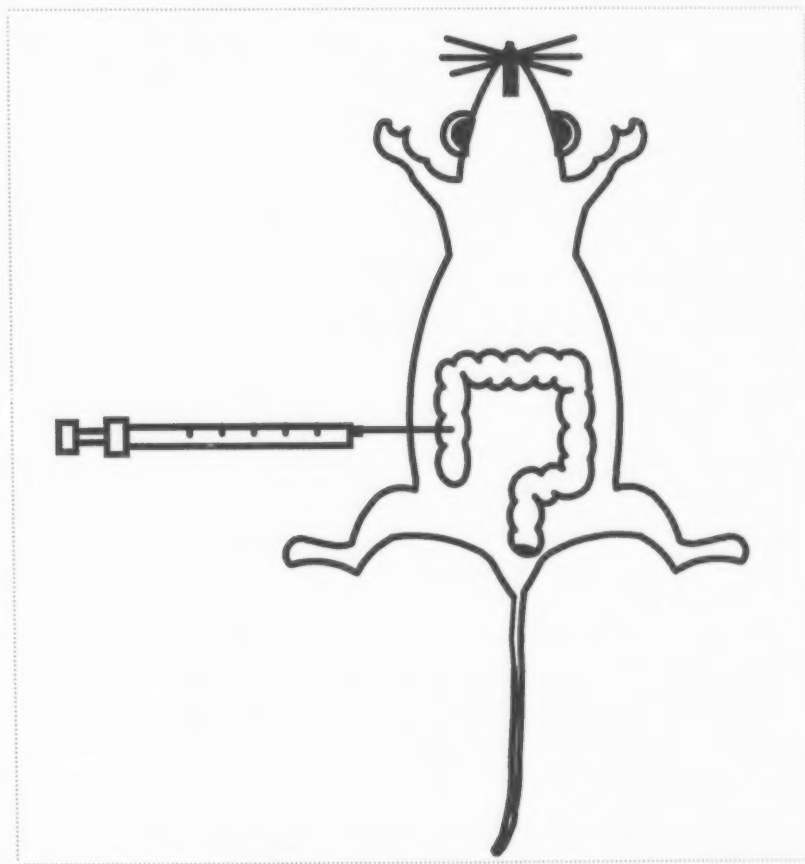


Figure 4-1. Induction of colitis.

1. One tie at ceacal end of the colon
2. Luminal content flushed out with saline
3. 4% acetic acid injected into the colon
4. Acetic acid flushed out with air

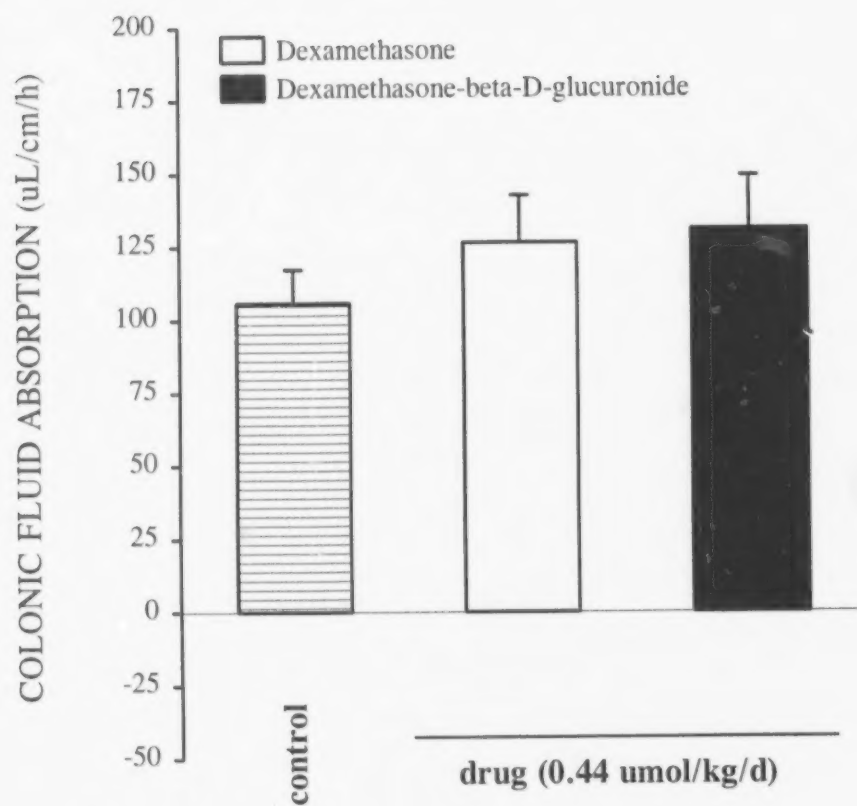


Figure 4-2. Effects of dexamethasone and its glucuronide prodrug on in vivo colonic fluid absorption in control rats (n=6).

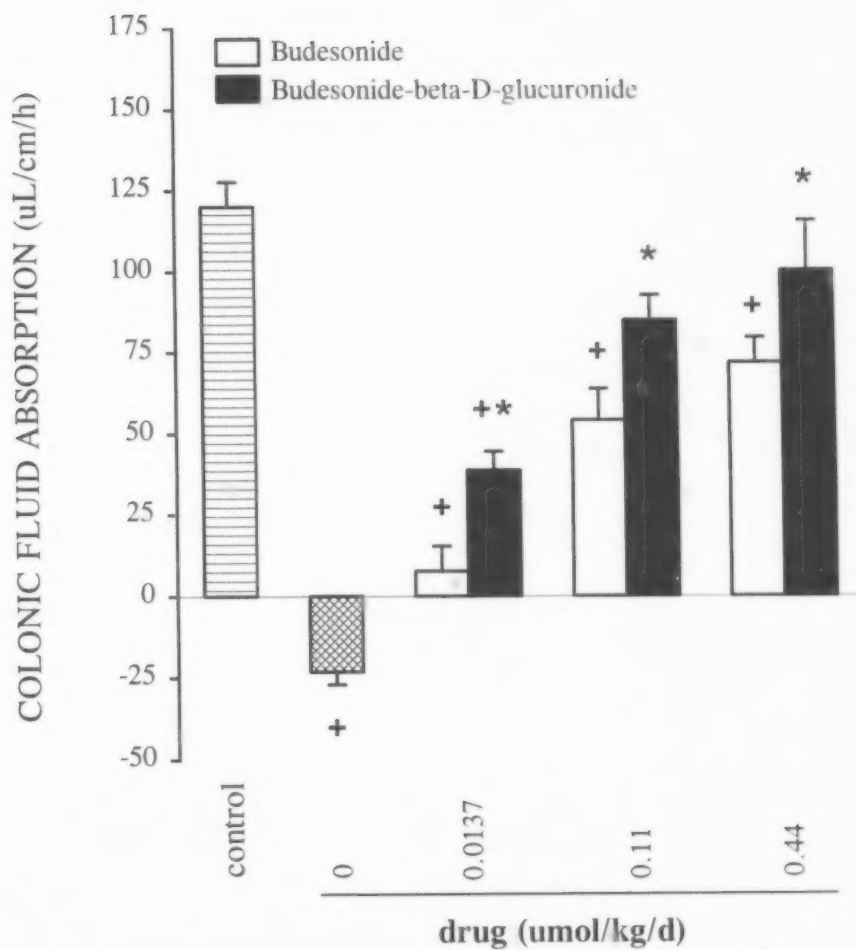


Figure 4-3. Effects of budesonide and its glucuronide prodrug on in vivo colonic fluid absorption in colitic rats (n=6).

* p < 0.02 compared with free budesonide.

+ p < 0.01 compared with control.

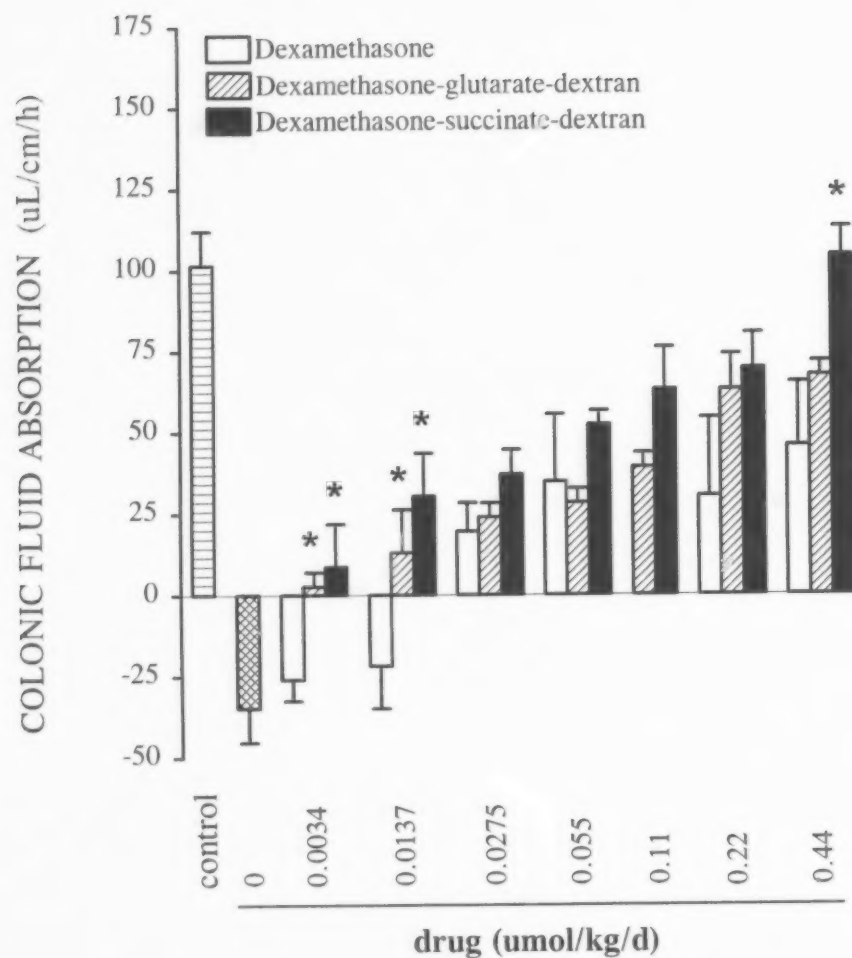


Figure 4-4. Effects of dexamethasone and its dextran prodrugs on in vivo colonic fluid absorption in colitic rats (n=6).
* $p < 0.02$ compared with free dexamethasone.

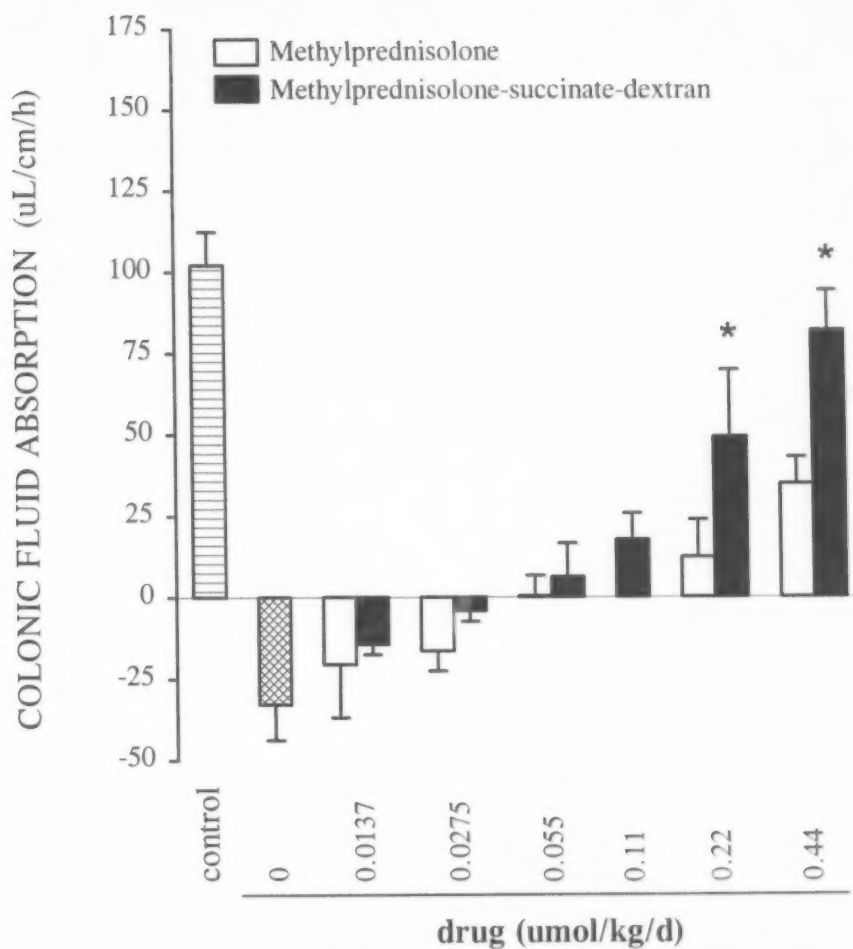


Figure 4-5. Effects of methylprednisolone and its dextran prodrug on in vivo colonic fluid absorption in colitic rats (n=6).
* p < 0.02 compared with free methylprednisolone.

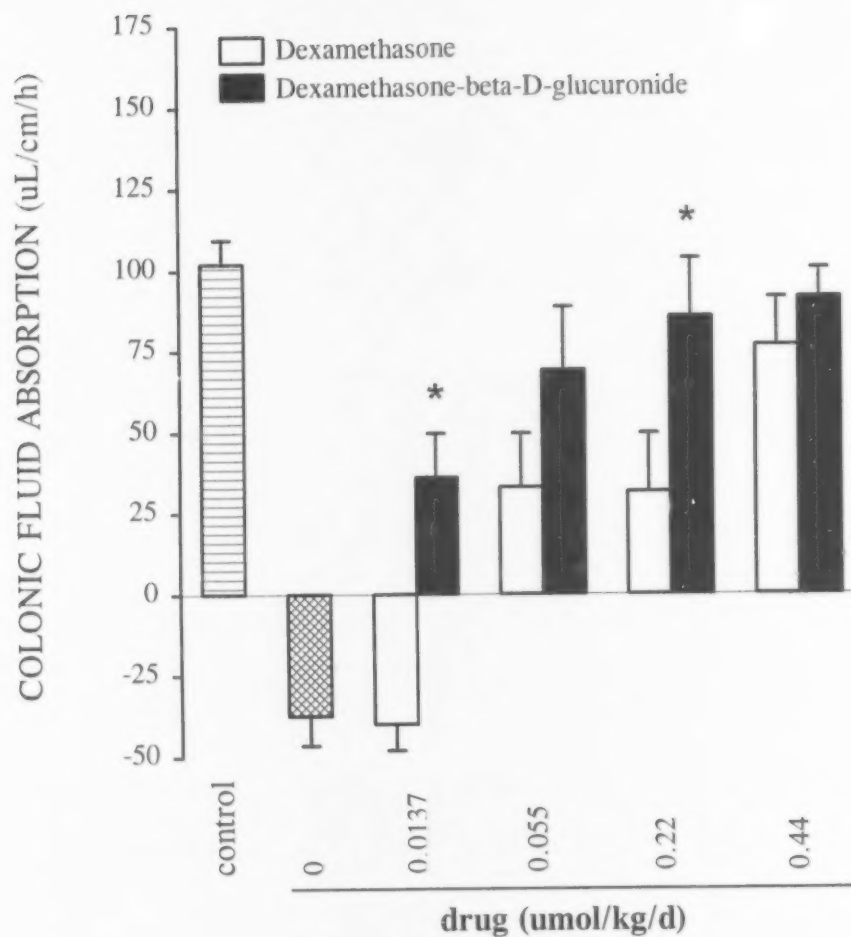


Figure 4-6. Effects of dexamethasone and its glucuronide prodrug on in vivo colonic fluid absorption in colitic rats (n=6).
* $p < 0.05$ compared with free dexamethasone.

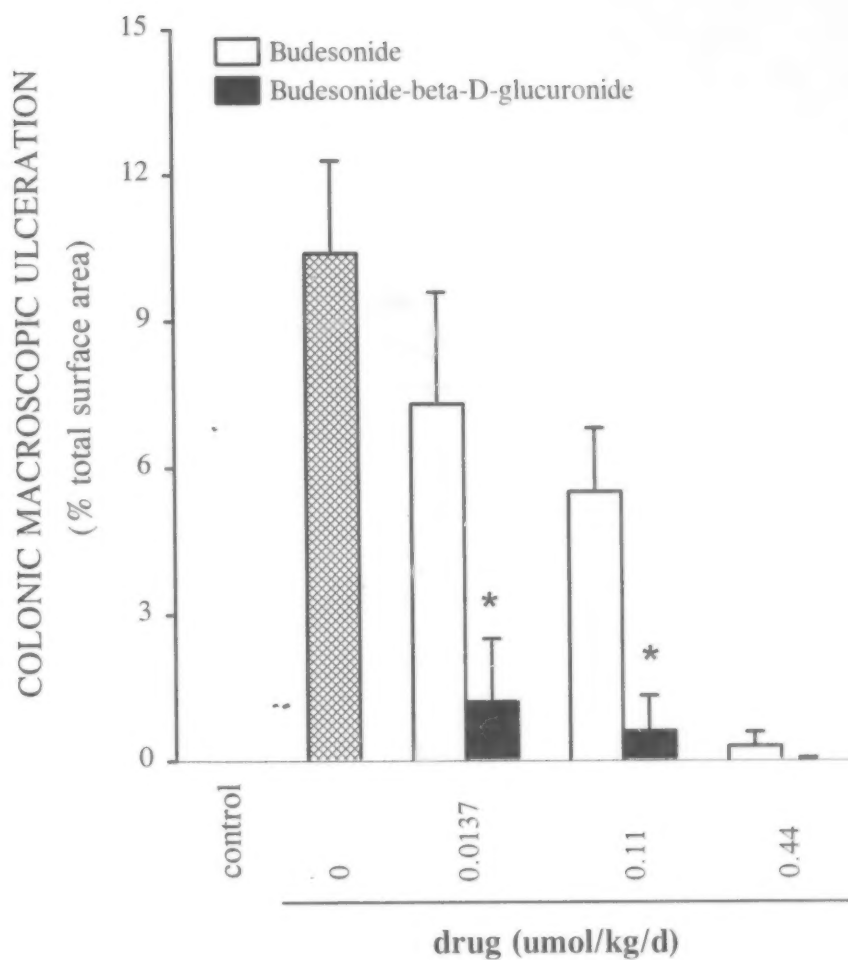


Figure 4-7. Effects of budesonide and its glucuronide prodrug on colonic macroscopic ulceration in colitic rats (n=6).

* $p < 0.02$ compared with free budesonide.

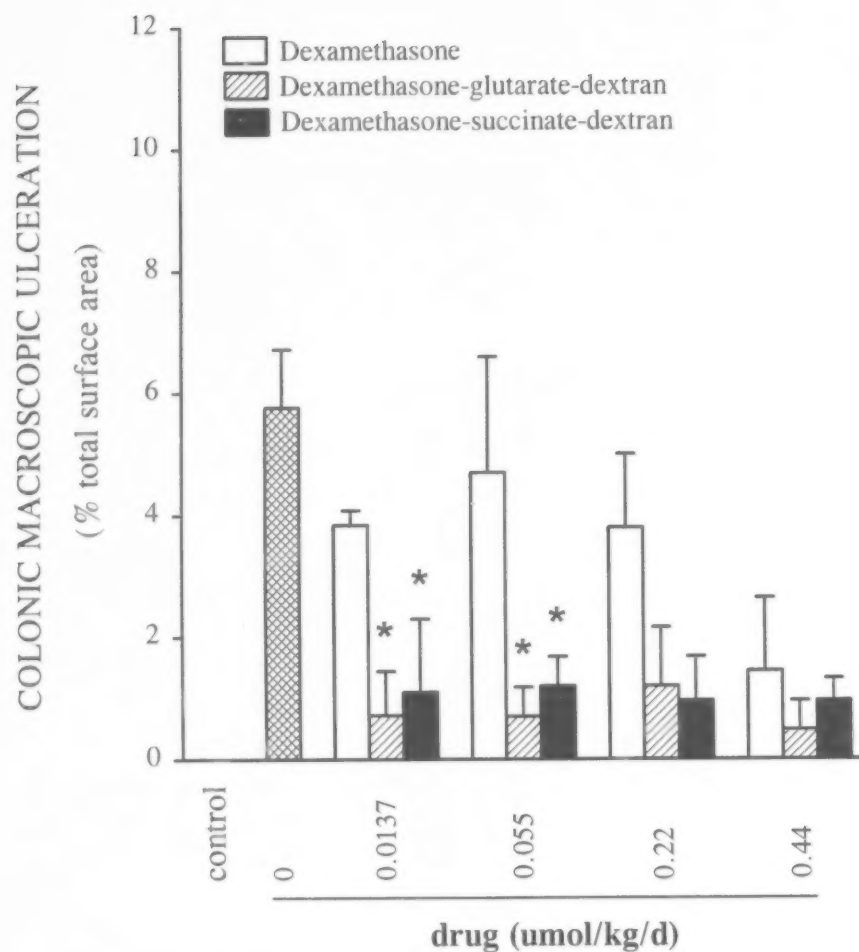


Figure 4-8. Effects of dexamethasone and its dextran prodrugs on colonic macroscopic ulceration in colitic rats (n=6).

* $p < 0.02$ compared with free dexamethasone.

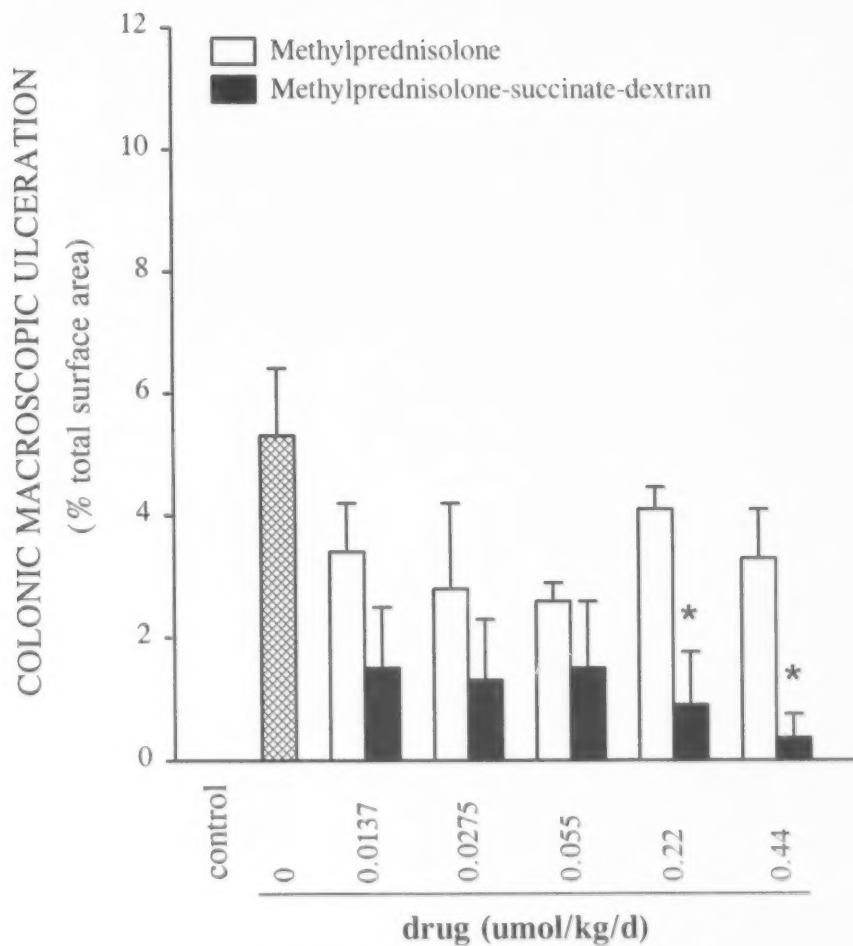


Figure 4-9. Effects of methylprednisolone and its dextran prodrug on colonic macroscopic ulceration in colitic rats (n=6).

* $p < 0.02$ compared with free methylprednisolone.

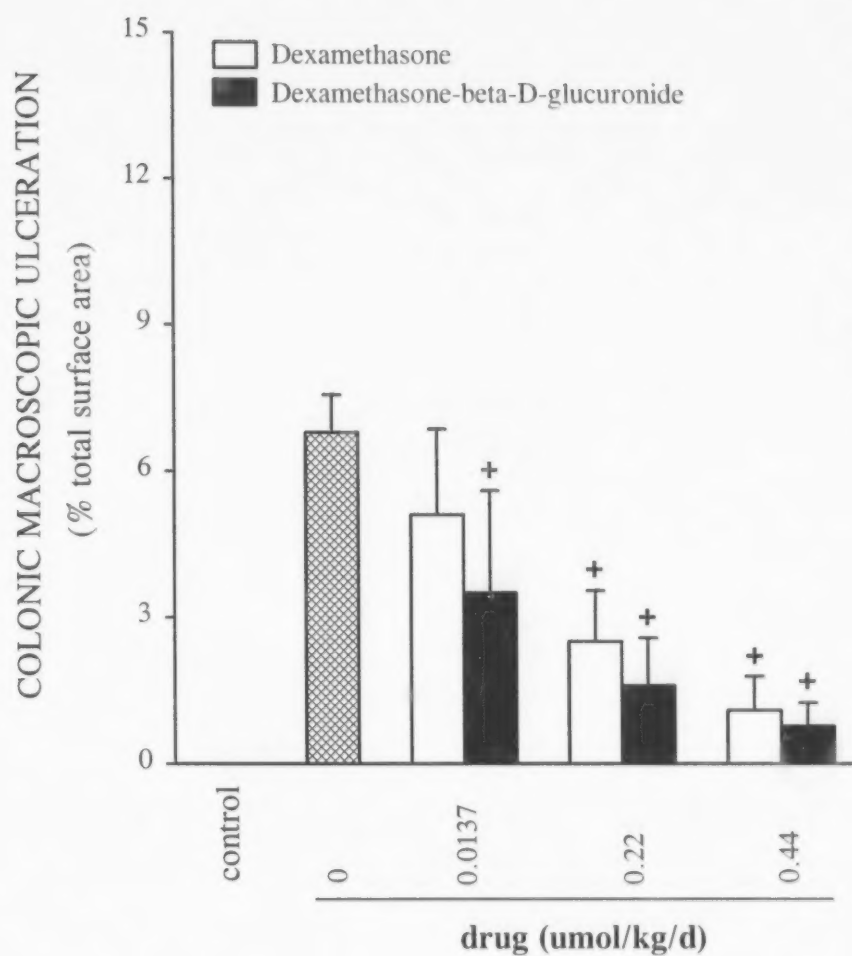


Figure 4-10. Effect of dexamethasone and its glucuronide prodrug on colonic macroscopic ulceration in colitic rats (n=6).
+p<0.01 compared with control.

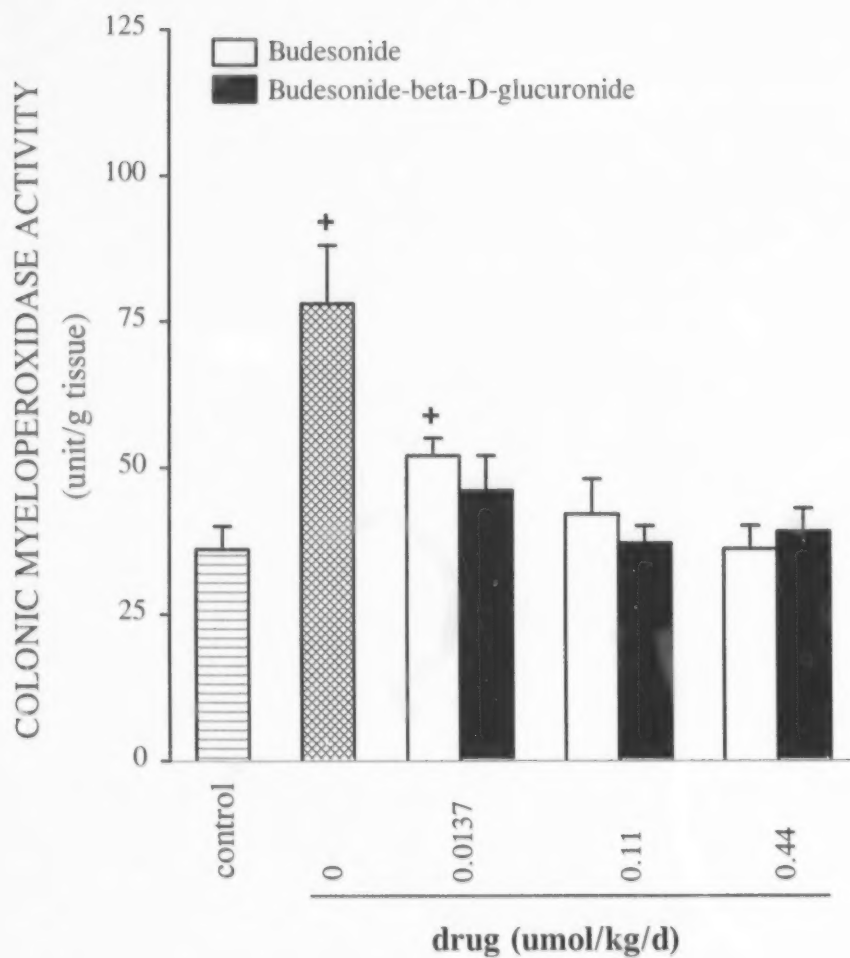


Figure 4-11. Effects of budesonide and its glucuronide prodrug on colonic myeloperoxidase activity in colitic rats (n=6).

*p < 0.05 compared with control.

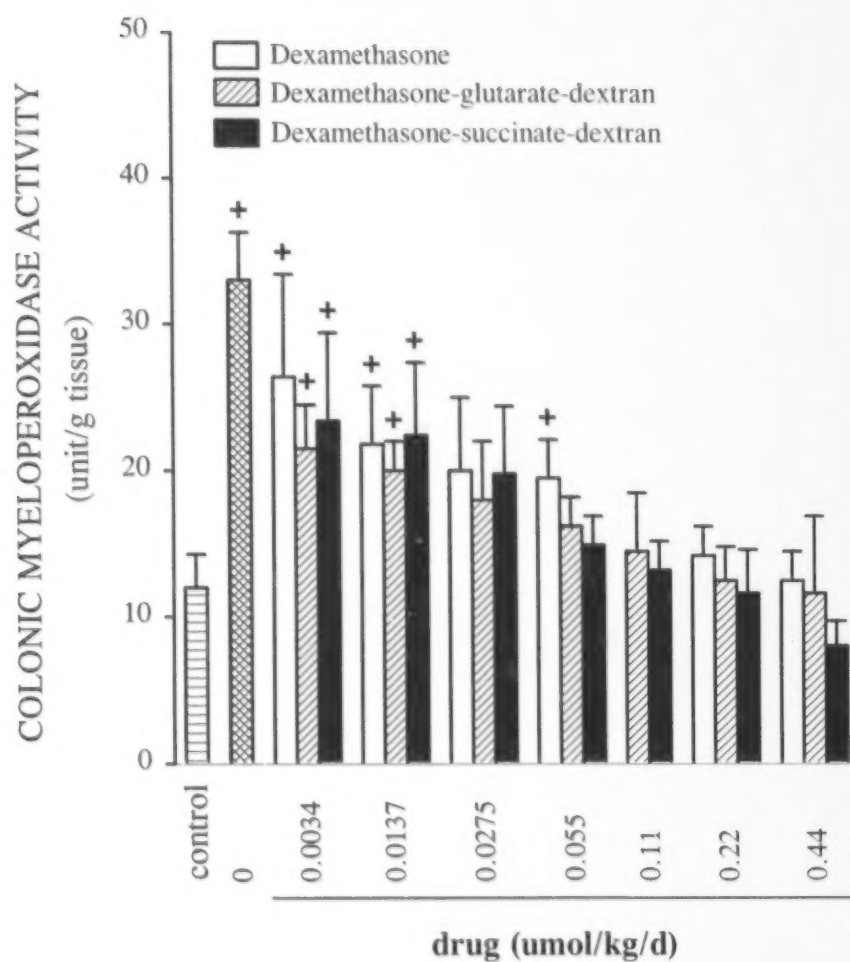


Figure 4-12. Effects of dexamethasone and its dextran prodrugs on colonic myeloperoxidase activity in colitic rats (n=6).
 *p < 0.02 compared with control.

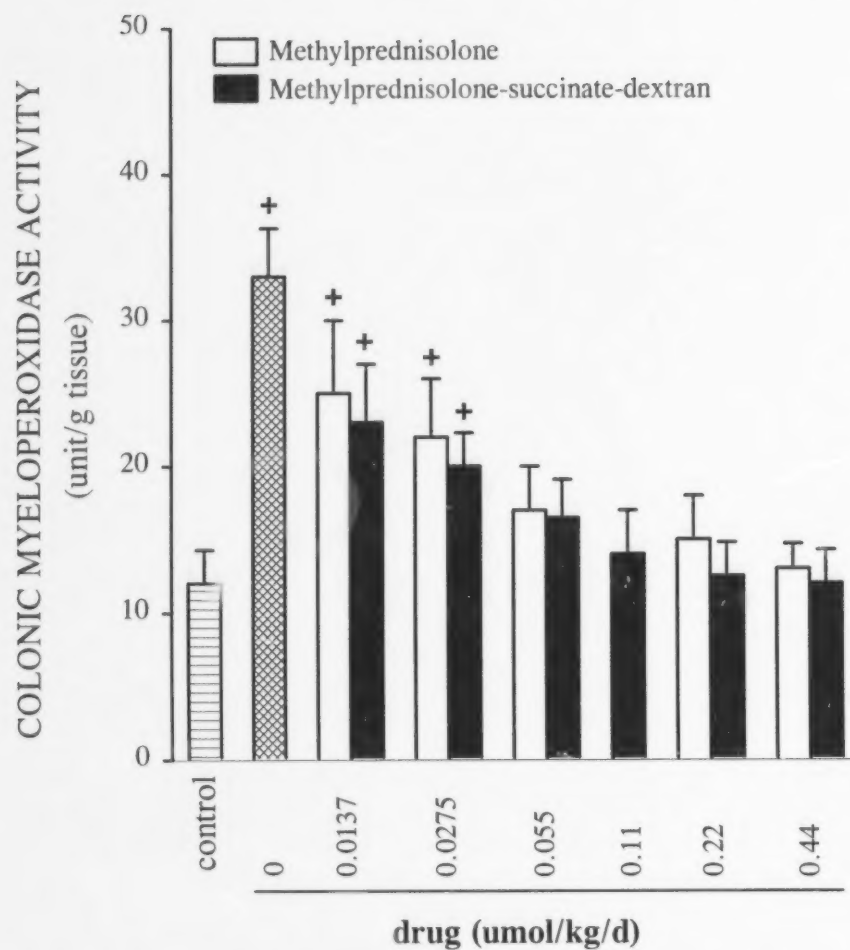


Figure 4-13. Effects of methylprednisolone and its dextran prodrug on colonic myeloperoxidase activity in colitic rats (n=6).
* $p < 0.02$ compared with control.

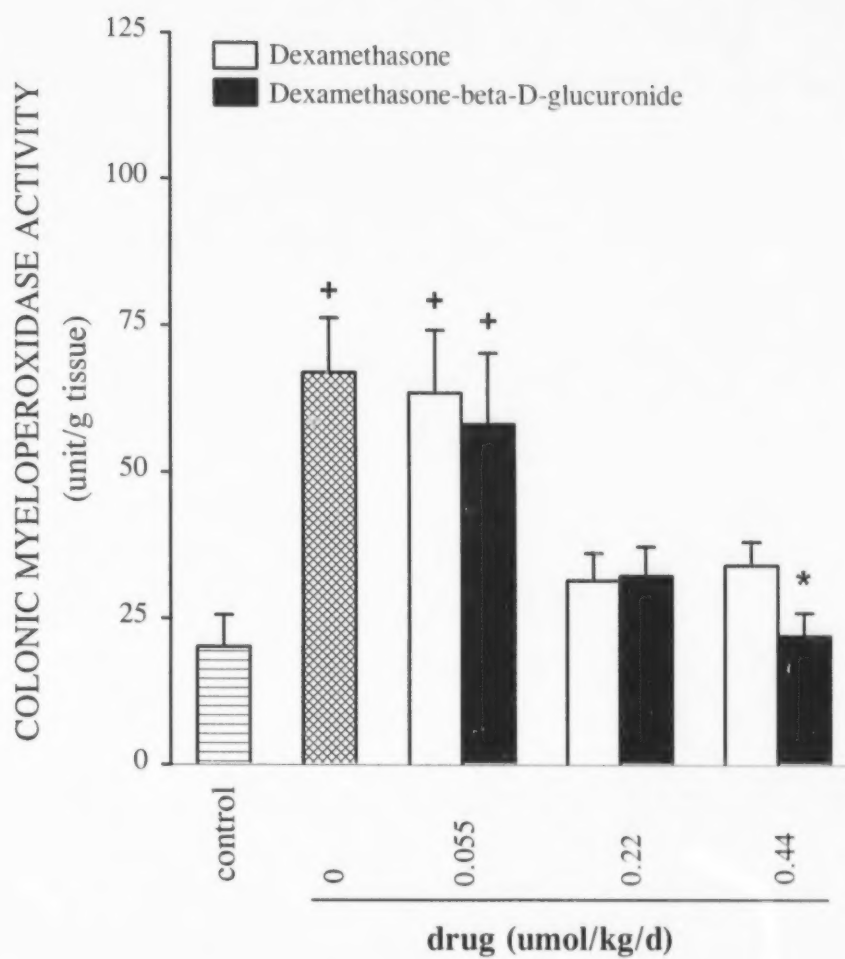


Figure 4-14. Effect of dexamethasone and its glucuronide prodrug on colonic myeloperoxidase activity in colitic rats (n=6).

* $p < 0.05$ compared with free dexamethasone

⁺ $p < 0.01$ compared with control.

CHAPTER 5

ADRENAL SUPPRESSION STUDIES

5.1 INTRODUCTION

The side-effects of glucocorticoids are related to high blood concentrations of the drugs. Since glucocorticoids exert their anti-inflammatory effect locally in IBD treatment, a prodrug delivery system which releases free glucocorticoids in the large intestine and minimizes systemic absorption of the free drugs would be ideal for increasing efficacy and reducing side-effects. In previous studies it was shown that in the colon specific bacterial enzymes hydrolyze the bond connecting a drug and its carrier molecule (1-2). A higher colonic luminal free drug concentration and a lower blood free drug concentration were attained. McLeod et al tested the stability of dexamethasone-succinate-dextran (DS-dextran), dexamethasone-glutarate-dextran (DG-dextran), and methylprednisolone-succinate-dextran (MPS-dextran) prodrugs in buffer solution and in the GI tract (3). In buffer solution the chemical degradation half-life was 75 hours for DS-dextran, 103 hours for DG-dextran, and 82 hours for MPS-dextran. Incubation of dexamethasone-21-hemiesters with rat GI content revealed that the hemiester is hydrolyzed throughout the GI tract, but the highest esterase activity was in the small intestine (2). When the dextran prodrugs were incubated with the contents of different intestinal segments, glucocorticoid-dextran conjugates resisted breakdown in the presence of a high level of esterase in the contents of the small intestine but were readily hydrolyzed in the contents of the caecum and colon. These results indicate that dextran protects both ester bonds from hydrolysis by esterases in the small intestine. McLeod et al also performed a pharmacokinetic study on glucocorticoid-dextran prodrugs. Using intragastric infusion, it was found that blood concentrations of DX were significantly lower after intragastric administration of the dextran conjugates (DS-dextran and DG-dextran) compared to those after the administration of free DX. The concentrations of free DX were significantly higher in caecum and colon tissue and significantly lower in small intestine tissue after

intra-gastric administration of dextran prodrugs (3).

Haeberlin et al examined the stability of another prodrug, dexamethasone- β -D-glucuronide (DX-glrd), in GI tract contents (2). After incubating the prodrug with the supernatants of intestinal contents and mucosa of different intestinal segments from conventional, germ-free and colitic rats, the specific activity of luminal β -D-glucuronidase in the caecum and colon was found to be 20- to 80-fold greater than that in the distal small intestine of conventional and colitic rats, respectively. Although the enzyme activity was steadily increased from the proximal small intestine to the large intestine, no significant change was observed. Compared to conventional and colitic rats, germ-free rats had very little luminal β -D-glucuronidase activity, confirming that the hydrolytic enzyme is produced by intestinal bacteria. The luminal β -D-glucuronidase activity in the intestinal mucosa was about 2-fold higher in conventional and colitic rats than in germ-free rats but, generally, it was very low in the mucosa from all three groups. Furthermore, the permeability of a Caco-2 monolayer was found 20-fold higher for free DX than for DX-glrd prodrug, suggesting that the steric structural characteristics of the conjugate hindered the absorption of the prodrug.

Based on these studies it is reasonable to predict that a DX-glrd prodrug would retain more DX in the lumen of the colon than would be the case using free DX, resulting in the lower blood concentrations of DX after prodrug administration, leading to lower hypothalamic-pituitary-adrenal axis suppressive effects.

This chapter describes experimental data that addresses the distribution of orally-administered free DX and DX-glrd in the lumen of GI tract and in the blood. The effects of free corticosteroids and respective prodrugs on blood concentrations of ACTH and corticosterone are also examined.

5.2 MATERIAL AND METHODS

In this study all the materials and methods are same as described in chapter 4, except where otherwise stated.

5.2.1 DX and DX-glrd distribution studies

Triamcinolone acetonide and β -D-glucuronidase (type IX from *E. Coli*) were obtained from Sigma Chemical Co. (St. Louis, MO).

5.2.1a Sample Preparation

Normal rats ($n=3$) were dosed between 8:00 and 9:00 AM. DX or DX-glrd ($3.7 \mu\text{mol/kg}$) were administered by oral gavage in 1.0 mL water/ethanol solution (9:1, v/v). At appropriate time points (1, 2.5, 3.5, 4.5, 6 and 9 hours) the rats were anaesthetized by i.p administration of 0.6 mL of sodium pentobarbital (69.8 mg/ml) then 7-8 mL of blood were withdrawn by intracardiac puncture. The blood was heparinized and plasma separated by centrifugation (8 min, 5°C , $2500 \times g$) which was then stored at -30°C . Immediately following collection of blood, the GI tract was isolated, divided into stomach, proximal small intestine (PSI), distal small intestine (DSI), cecum, and colon. The contents of each segment were removed by gently squeezing. The contents and tissues were placed in sample tubes or small plastic bags, quickly frozen and stored at -30°C until HPLC analysis. For analysis of luminal contents, the frozen samples were thawed, weighed, and diluted with chilled (4°C) 0.075 M potassium phosphate buffer (pH 6.8) to 6 to 20 ml depending on the amount of luminal content. The suspended contents were homogenized using an Ultra-Turrax homogenizer (type TP18/10SI, 20,000 rpm, Janke & Kunkel, IKA Werk, Staufen, Germany). Samples were kept on crushed ice during homogenization, which took approximately 30 seconds. To prepare homogenates of mucosal scrapings, the intestines were cut open longitudinally, placed on a glass plate and the mucosa scraped with the edge of a microscope slide. The samples were then homogenized as described for the luminal contents. The homogenates were well mixed once again and aliquots (two for DX treated animals and two for DX-glrd treated animals) of 1.00 mL were pipetted into glass samples tubes (10 mL). Duplicate samples of the DX-glrd treated rats were each mixed with $50 \mu\text{L}$ of β -D-glucuronidase solution (1mg/mL in water) and incubated overnight at 37°C . The samples were then mixed with $25 \mu\text{L}$ of an ethanolic solution of the internal standard triamcinolone acetonide ($40 \mu\text{g/mL}$) and 1.0 mL

of saturated sodium chloride and extracted twice with 4 mL of methyl-t-butyl ether/pentane (6:4, v/v). The combined organic extracts were washed with 1mL 0.1N NaOH solution followed by 1.0 mL water. The extracts were then evaporated to dryness under argon (Meyers-N-Evaporator) and the dried residues maintained at -30°C until required analysis of glucuronide by HPLC. Prior to analysis by HPLC, the residues were reconstituted in 120 μ L of acetonitrile/water (1:1, v/v), centrifuged (5 min, at top speed, Eppendorf 5412 centrifuge) and 80 μ L of the supernatant were injected.

5.2.1b HPLC Analysis

DX levels were determined by reversed phase HPLC. The chromatographic system consisted of the following components (all from Waters Associates, Inc., Milford, MA): a high pressure pump (model 6000A), an automatic sample injector (WISP710B), a UV-absorption detector (model 481), and a chromatographic data station (model 840). The separation was performed on a Whatman partisil ODS-3 column (10 μ m, 3.9 x 300mm). A mobile phase at a flow rate of 1.0 mL/min was used. The mobile phase consisted of 0.02M sodium acetate buffer (pH 4.8)/acetonitrile (68:32, v/v). All separations were performed at ambient temperature. The retention times observed under these conditions were 14.5 minutes (DX) and 18 minutes (triamcinolone acetonide). DX-glrd eluted with the solvent front. Calibration curves were obtained either by direct injection (15-30 μ L) of ethanolic DX and DX-glrd solutions (concentration of DX-glrd external standards, 5-100 μ L/ml) or by injection of mixtures of varying amounts of dexamethasone with internal standards. The calibration curve prepared in the range of 100-4000 ng DX (amount of drug in 120 μ L reconstituted sample) was linear ($r > 0.999$) with respect to ratios (DX/internal standard) of peak areas and peak heights. The ratios of the peak areas were used for the calculations (2).

5.2.2 Effect of Glucocorticoids and Their Prodrugs on Serum Corticosterone

After colitis was induced in the rats, drugs and their prodrugs were administered as described as above. After the rats (n=6) were given a pentobarbital overdose, blood (2 mL) was collected into an evacuated blood collection tube (Becton Dickinson

Vacutainer Systems, Rutherford, NJ, USA) between 0800 and 1000 AM by intracardiac puncture. The blood was then centrifuged at 4°C for 10 min at 4,000 rpm (Centra-7, International Equipment Co., Needham Heights, MA, USA) and the serum removed and stored at -70°C prior to analysis. Stock anti-corticosterone antiserum (B3-163) was obtained from Endocrine Sciences RIA Reagents, (Tarzana, CA, USA). Diluted antiserum was prepared as follows: 10 mL borate buffer (0.05M, pH 8); 400,000 dpm of 1,2-³H-corticosterone; 0.2 mL of 10% of bovine serum albumin in borate buffer; 0.2 mL of 0.25 % bovine gamma globulin in borate buffer; and 100 µL of stock antiserum. The serum sample for analysis was thawed and placed in a 12 x 75 mm test-tube, diluted with borate buffer and incubated at 60°C for 30 min. The solution was transferred to a 2 mL conical tube (Kimax #45150, Fisher Scientific, Nepon, ON, Canada) and incubated with 4 µL diluted antiserum at 37°C for 45 min then at room temperature for 2 hr. Saturated ammonium sulphate solution (0.25 mL) was added to each tube and the mixture was centrifuged at 3,000 rpm for 10 minutes to separate free and bound steroid. The supernatant was decanted into a microvial (Simport Ltd., Quebec City, QB, Canada), 4 mL of scintillation cocktail was added (Scintiverse, bio-HP, Fisher Scientific, Nepon, ON, Canada) and radioactivity was counted on a liquid scintillation counter (LKB Wallace, 1219 Rack Beta, Turku, Finland).

5.2.3 Effect of Glucocorticoids and Their Prodrugs on Plasma ACTH

Adrenocorticotrophic hormone (ACTH) was determined in blood (3 mL) collected immediately after sacrificing the rats by intracardiac puncture. Blood was drawn into an chilled siliconized tube. The blood was then centrifuged at 4°C for 10 min at 4,000 rpm. The plasma was transferred to a polypropylene tube and stored at -70°C until analysis. The sample did not come into contact with glass at any time throughout the sample collection procedure. ACTH was measured using a commercial immunoassay kit (Nichols Institute, San Juan, Capistrano, CA, USA). The ACTH immunoassay incorporates a monoclonal antibody and a polyclonal antibody, both with high affinity and specificity for defined amino acid regions of the ACTH molecule. The polyclonal antibody is prepared

by affinity chromatography to bind only to the C-terminal region of ACTH. The monoclonal antibody binds only to the N-terminal region of ACTH. Both antibodies bind ACTH without competition or steric interference from each other, and form a soluble sandwich complex. The monoclonal antibody is radiolabelled for detection, while the polyclonal antibody is coupled to biotin. The addition to the reaction mixture of an avidin-coated plastic bead allows for a specific and efficient means of binding the sandwich complex via the high affinity reaction between biotin and avidin. In the assay, standards, controls and samples were incubated with a solution containing both the radiolabelled antibody and biotin coupled antibody and an avidin-coated plastic bead. At the end of the incubation period, the bead was washed to remove unbound components and the radioactivity bound to the solid phase was measured in a gamma counter (Packard, Fisher Scientific, Nepon, ON, Canada). Since the complex occurs only in the presence of an intact ACTH molecule containing both N-terminal and C-terminal regions, the radioactivity of the bead bound complex is directly proportional to the amount of intact ACTH in the sample.

5.2.4 Statistics

Data are expressed as means $\pm$ SD. Nonparametric analysis of the dose-response curves was performed using the Kruskal-Wallis test. Statistical analysis of the data was performed by repeated-measure analysis of variance. When the overall analysis showed significance, the Students t-test was used to examine the location and significance of differences.

5.3 RESULTS

5.3.1 DX and DX-glrd Distribution Studies

Following oral administration of the drug and its prodrug to control rat, the recovery of DX in the colonic luminal contents of the rat after gastric intubation of DX-glrd or free DX is shown in Table 5-1. In DX-glrd treated rat most DX was recovered between 2 and 6 hour after its administration. Total recovery of DX from DX-glrd-treated

rat was about 6 times that from the free DX-treated rats.

The distribution of DX along the small and large intestinal mucosa and luminal contents was determined at various times following oral administration of $3.7\mu\text{mol/kg}$ DX-glrd or same dose of free DX. In the small intestinal mucosa, there was little DX measured after the administration of free DX or DX-glrd (Figure 5-1). In small intestinal luminal contents, more DX was recovered in rats treated with DX-glrd (Figure 5-2). Little DX was recovered from the colonic mucosa after the administration of free DX or DX-glrd (Figure 5-3) whereas in the colonic luminal contents, relatively large amount of DX was recovered following the administration of DX-glrd (Figure 5-4). The amount of DX in colonic luminal contents in DX-glrd-treated rats was 8 times that in DX-treated rats.

Plasma concentrations of DX over time after oral administration of the prodrug DX-glrd ($3.7\mu\text{mol/kg}$) or free DX ($3.7\mu\text{mol/kg}$) are shown in Figure 5-5. In animals given prodrug the plasma peak concentration appeared at 3.5 h after administration of the prodrug. In animals given free DX the plasma peak concentration of DX appeared at 2.5 hr and the peak was twice that obtained from prodrug-treated rats. The plasma concentrations of DX in both groups became the same after 6 h following administration.

5.3.2 Effect of Drugs and Their Prodrugs on Serum Corticosterone Levels

5.3.2a BD and BD-glrd

Figure 5-6 shows serum corticosterone levels in budesonide-treated animals. In animals that had colitis induced with acetic acid but received no treatment, there was a significant elevation in serum corticosterone levels compared to controls ($p < 0.02$). Treatment with free BD significantly reduced corticosterone to subnormal levels at all doses tested ($p < 0.02$). In contrast, treatment with BD-glrd retained corticosterone levels at normal values, with no evidence of adrenosuppression even at the highest dose.

5.3.2b DX, MP and their dextran prodrugs

Figure 5-7 and Figure 5-8 show serum corticosterone levels in free drug- or dextran prodrug-treated rats. The levels were determined mainly at higher doses (0.22 and

0.44 $\mu\text{mol/kg/d}$) where adrenosuppression was expected to occur. The corresponding effects were determined for the DX compounds at dose of 0.0137 $\mu\text{mol/kg/d}$. In rats with colitis but received no glucocorticoid treatment, there was a significant elevation in serum corticosterone levels compared to controls ($p < 0.01$). As seen in Figure 5-7, treatment with free dexamethasone significantly reduced serum corticosterone to subnormal levels ($p < 0.05$). In contrast, treatment with the DG-dextran and DS-dextran prodrug conjugates retained corticosterone levels at normal values, with no evidence of adrenosuppression even at the highest dose. Figure 5-8 shows that free MP suppressed corticosterone level at dose of 0.22 $\mu\text{mol/kg/d}$ but MPS-dextran did not have suppressive effect except at the highest dose.

5.3.2c DX and its DX-glrd

As shown in Figure 5-9, the serum corticosterone level was decreased in a dose-dependent manner. The prodrug did not suppress the serum corticosterone level except at the highest doses. However free DX suppressed the corticosterone level at the lowest dose, indicating a higher concentration of free drug in the circulation.

5.3.3 Effect of Glucocorticoids and Their Prodrugs on Plasma ACTH Levels

5.3.3a BD and BD-glrd

Data shown in Figure 5-10 demonstrate the effect of BD on plasma ACTH levels. The levels were comparable in both controls and rats with colitis. Similar to the corticosterone results, treatment with free BD significantly reduced plasma ACTH levels whereas BD-glrd did not.

5.3.3b DX, MP, and their dextran prodrugs

Data shown in Figure 5-11 and Figure 5-12 demonstrate the effect of DX and MP on plasma ACTH levels. The levels were elevated in animals with non-treated colitis compared to controls ($p < 0.01$). Similar to the corticosterone results, treatment with the free glucocorticoids significantly reduced plasma ACTH levels whereas their respective dextran conjugates (with the exception of DG-dextran) did not.

5.3.3c DX and DX-glrd

As shown in Figure 5-13, free DX severely suppressed the plasma ACTH level at the lowest dose ($0.0137 \mu\text{mol/kg/d}$). In contrast, DX-glrd did not show ACTH suppression effect even at the highest dose ($0.44 \mu\text{mol/kg/d}$).

5.4 DISCUSSION

Prodrugs for colonic delivery should be nonabsorbable from the stomach and small intestine and conversion to the active drug should occur mainly in the colon at a controlled rate. Recent studies on DX-glrd in rats under *in vitro* conditions indicated that this novel prodrug is relatively stable in the upper GI tract and is subject to quantitative hydrolysis by β -D-glucuronidase from intestinal bacteria in the caecum and colon (2). DX-glrd is a water soluble compound having a permeability coefficient across human Caco-2 cell monolayer equal to polyethylene glycol 4000 (2). Hence human intestinal epithelial membrane is relatively impermeable DX-glrd. The sharp gradient in β -D-glucuronidase activity in the rat lower GI tract enables the free DX to be released from DX-glrd at this site.

The distribution of DX and DX-glrd indicated that significantly more free DX was delivered to lumen of the rat large colon from DX-glrd compared to that of free DX. A larger amount of DX was recovered from the colonic mucosa when the prodrug (DX-glrd) was administered compared with that from administration of free DX. The amount of DX measured in plasma following oral administration of DX-glrd prodrug was about one third that following oral administration of DX. In other words, the systemic availability of DX from the prodrug is about one third that relative to orally-administered free DX. In a pharmacokinetic study on glucocorticoid-dextran prodrugs McLeod et al found that the relative bioavailability was 0.28 for DS-dextran and 0.16 for DG-dextran (3). Although bioavailability studies were not performed the results show that the total recovery of DX-glrd in the colon was 95%, indicating that the bioavailability of DX-glrd is very low. Because glucocorticoids exert their anti-inflammatory action locally and high

concentrations of glucocorticoids in blood will suppress the adrenal function, the low bioavailability of prodrugs has the advantages of decreased side-effect and increased efficacy.

Glucocorticoids suppress ACTH secretion from the pituitary by a feedback mechanism via the glucocorticoid receptor, since the receptor has been isolated from the pituitary gland. It has been found that the level of mRNA coding for ACTH in the pituitary increased more than 3-fold following adrenalectomy and decreased following dexamethasone administration (4). This suppressive effect on ACTH production is only seen in glucocorticoids because aldosterone did not change ACTH mRNA levels.

Adrenal suppression is directly related to blood levels of exogenous free glucocorticoid (5). In humans, ACTH directly regulates the production of cortisone, whereas in the rat ACTH regulates the production of corticosterone (6). Thus, measurement of ACTH and corticosterone is a sensitive means of determining the degree of suppression on hypothalamic-pituitary-adrenal (HPA) axis in rats.

BD is a newly synthesized glucocorticoid which possesses high local anti-inflammatory activity but with 90% first-pass hepatic metabolism (7). In order to increase anti-inflammatory efficacy and to reduce the HPA axis suppressive effects a controlled release capsule has been formulated to release free BD in the ileum. When administered orally inside the capsule to patients with active ileal Crohn's disease, BD (9 mg/d) was as efficacious as prednisolone (40 mg/d) in inducing disease remission after 10 weeks (8). Unfortunately, this dose of BD resulted in a 40% depression of plasma cortisol and ACTH despite its 90% first pass metabolism (8, 9). Results of pharmacokinetic and GI transit studies of orally-administered BD Controlled Ileal Release Capsules indicated that approximately 10% of the oral dose was available systemically and that less than 5% reached the colon (10). Therefore, it is unlikely that BD, and perhaps other glucocorticoids, in the same orally-administered Controlled Ileal Release Capsule will deliver active drug to the colonic lumen in sufficient quantities to be effective in treating colonic disease. Nevertheless, some healing of colonic disease may take place because

of the apparently high systemic availability of active glucocorticoids. Indeed, in animal studies BD administered by subcutaneous injection was able to heal experimentally induced colitis (11). But again, this route of administration has the danger of elevation of systemic levels of free glucocorticoids with concerns about HPA axis suppression.

In this study, free BD suppressed serum corticosterone at a very low dose whereas BD-glrd did not have a significant suppressive effect even at the highest dose. Although a significant difference between BD and its prodrug on the suppression of ACTH was calculated based on only one dose, groups treated at other doses showed a trend, suggesting that DX-glrd had less of a suppressive effect. Among the dextran prodrugs, except for DG-dextran at the highest dose, the glucocorticoid-dextran prodrug conjugates did not affect either ACTH or corticosterone levels at any of the doses tested. This is in contrast to free DX and MP which caused statistically significant adenosuppression. DX-glrd also produced lower suppressive effects on serum corticosterone and plasma ACTH levels compared to the free DX or MP treatment. These results are consistent with the low blood concentrations of free drugs after prodrug administration and relatively high blood concentrations of free drugs after free drug administration, observed in this study and described by others (3).

Serum corticosterone levels have been shown to correlate reasonably with plasma ACTH levels. Except for one dose of DX-glrd ($0.22 \mu\text{mol/kg/d}$), all the prodrugs showed a lower suppressive effect on corticosterone and ACTH than did the free drugs.

It is concluded that glucocorticoid prodrug conjugates can be administered orally to facilitate mucosal repair in rat colitis with significantly reduced adrenal suppression.

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Table 5-1. Amounts of drugs recovered from the colon following oral administration of DX and DX-glrd.

Time following oral administration* (h)	Percent drug recovered following DX-glrd administration	Percent drug recovered following free DX administration
0	0	0
1	0.2	0.8
2.5	14.1	6.3
3.5	34.2	2.7
4.5	18.2	1.4
6	27.2	3.2
9	2.6	0.5
Total drug recovered	96.5%	14.9%

*Free DX or DX-glrd was administered by oral gavage at 3.7 $\mu\text{mol/kg}$.

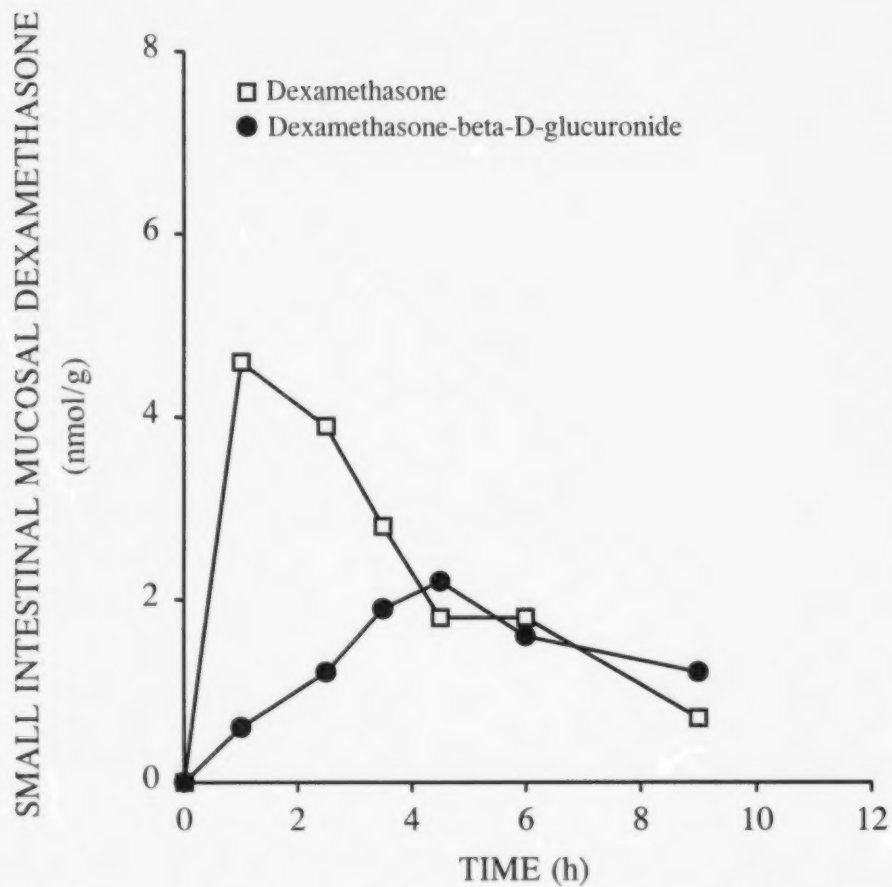


Figure 5-1. Small intestinal mucosal dexamethasone concentrations in control rats treated with dexamethasone or its glucuronide prodrug (n=3).

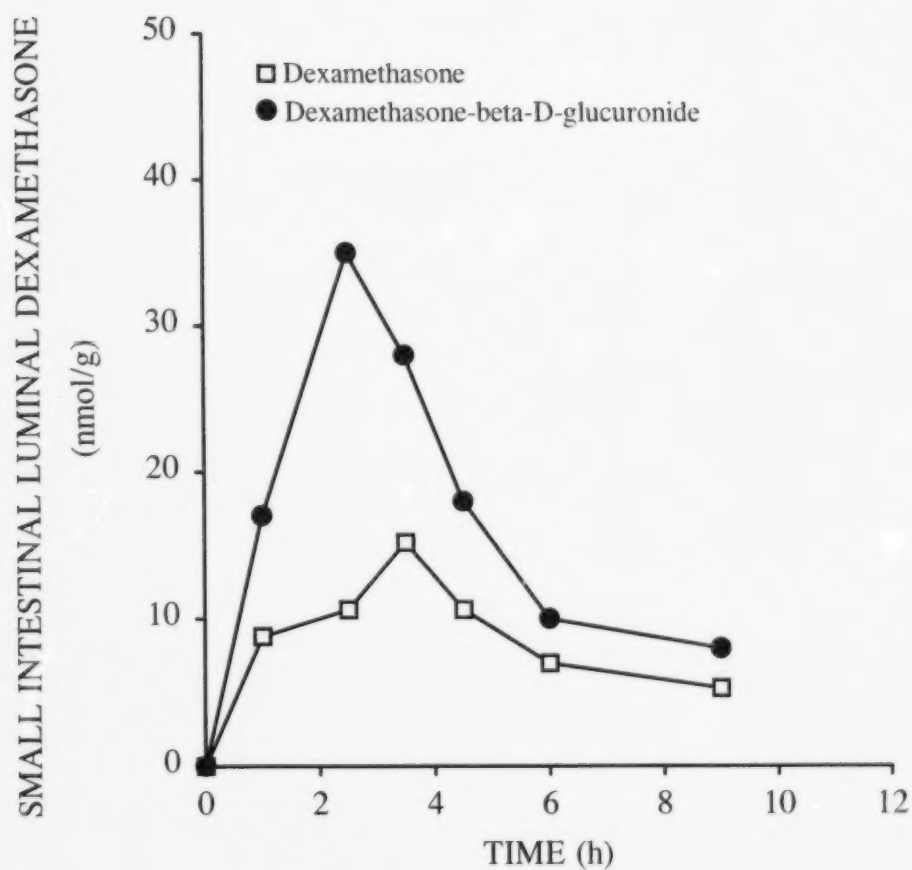


Figure 5-2. Small intestinal luminal dexamethasone concentrations in control rats treated with dexamethasone or its glucuronide prodrug (n=3).

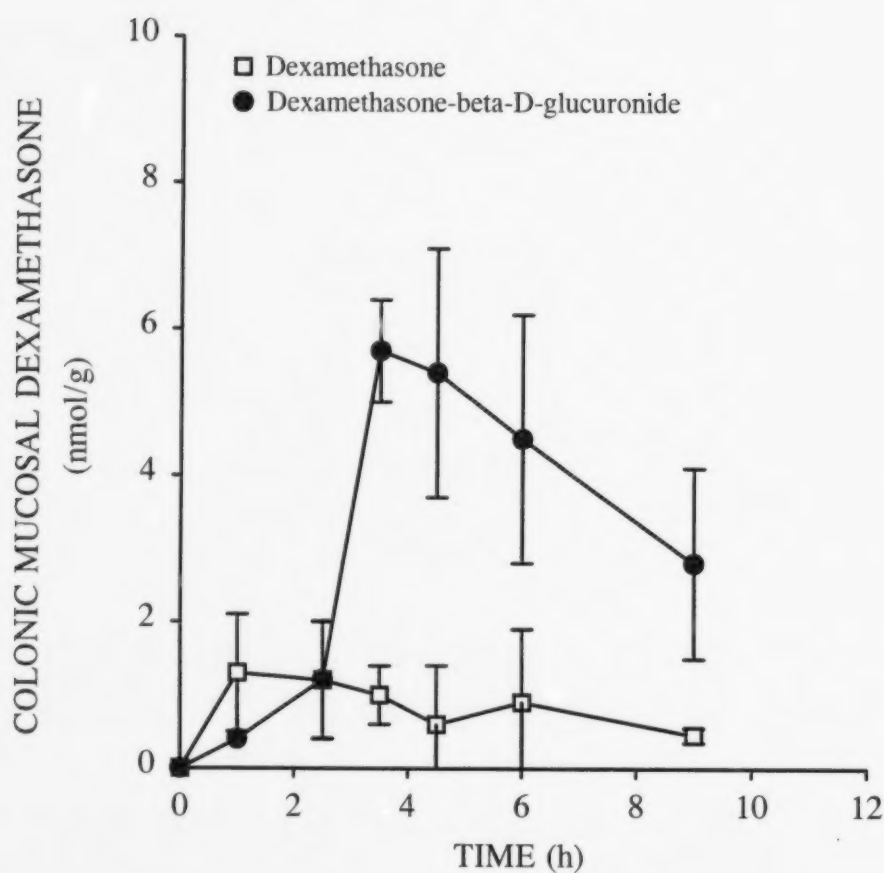


Figure 5-3. Colonic mucosal dexamethasone concentrations in control rats treated with dexamethasone or its glucuronide prodrug (n=3).

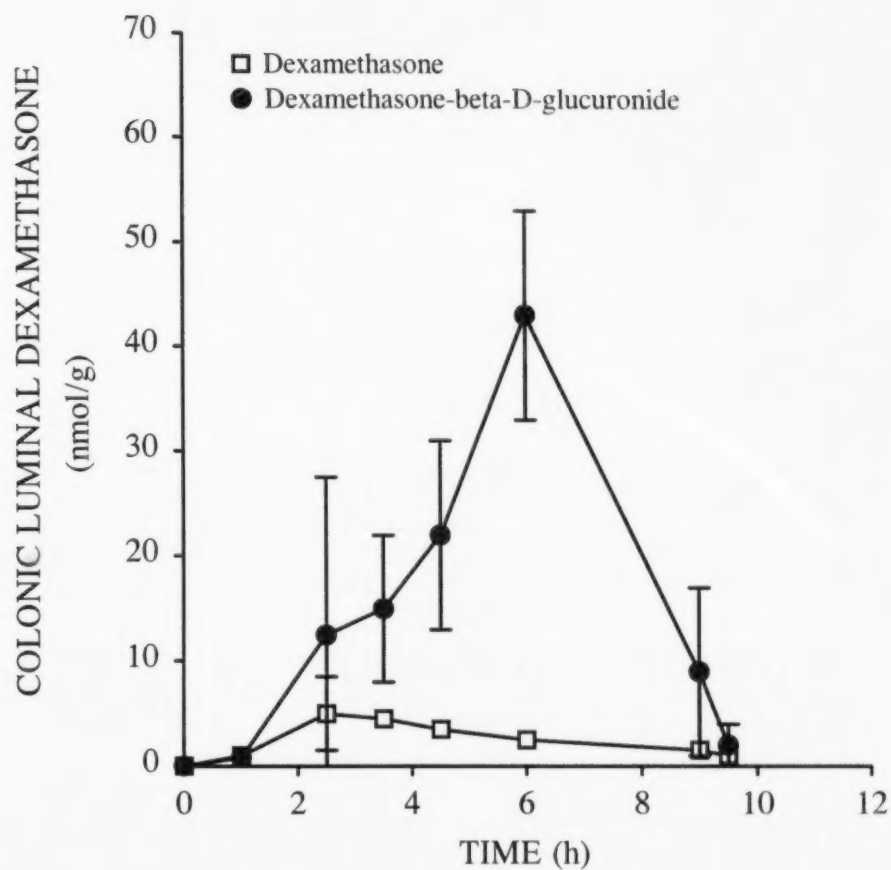


Figure 5-4. Colonic luminal dexamethasone concentrations in control rats treated with dexamethasone or its glucuronide prodrug (n=3).

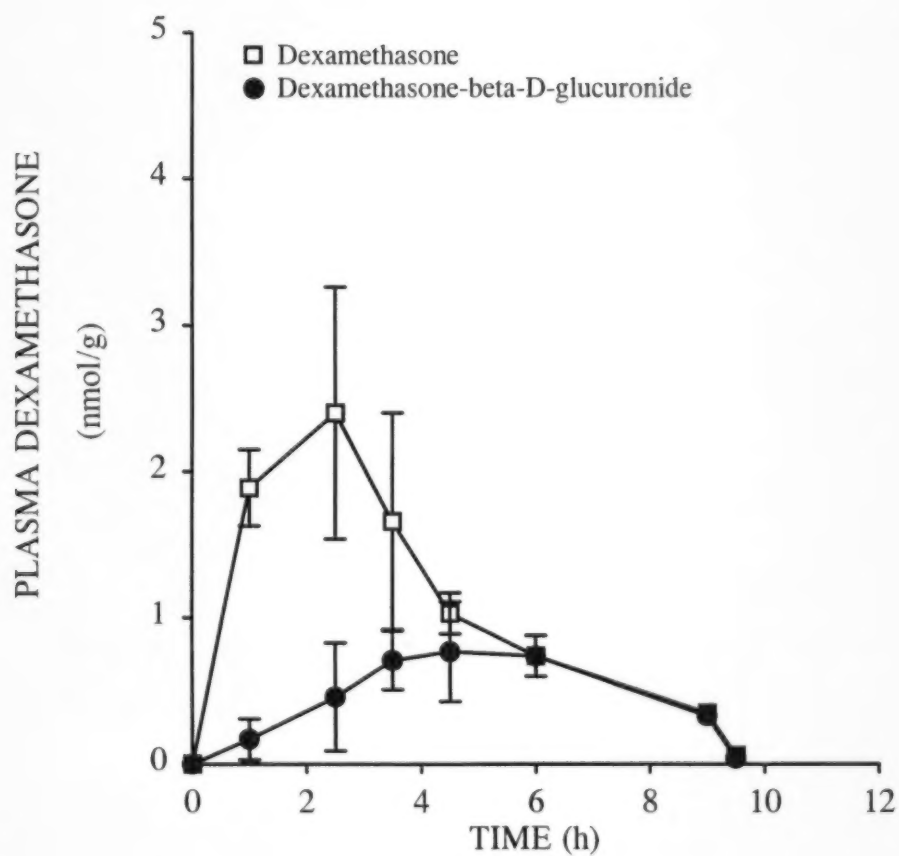


Figure 5-5. Plasma dexamethasone concentrations in control rats treated with dexamethasone or its glucuronide prodrug (n=3).

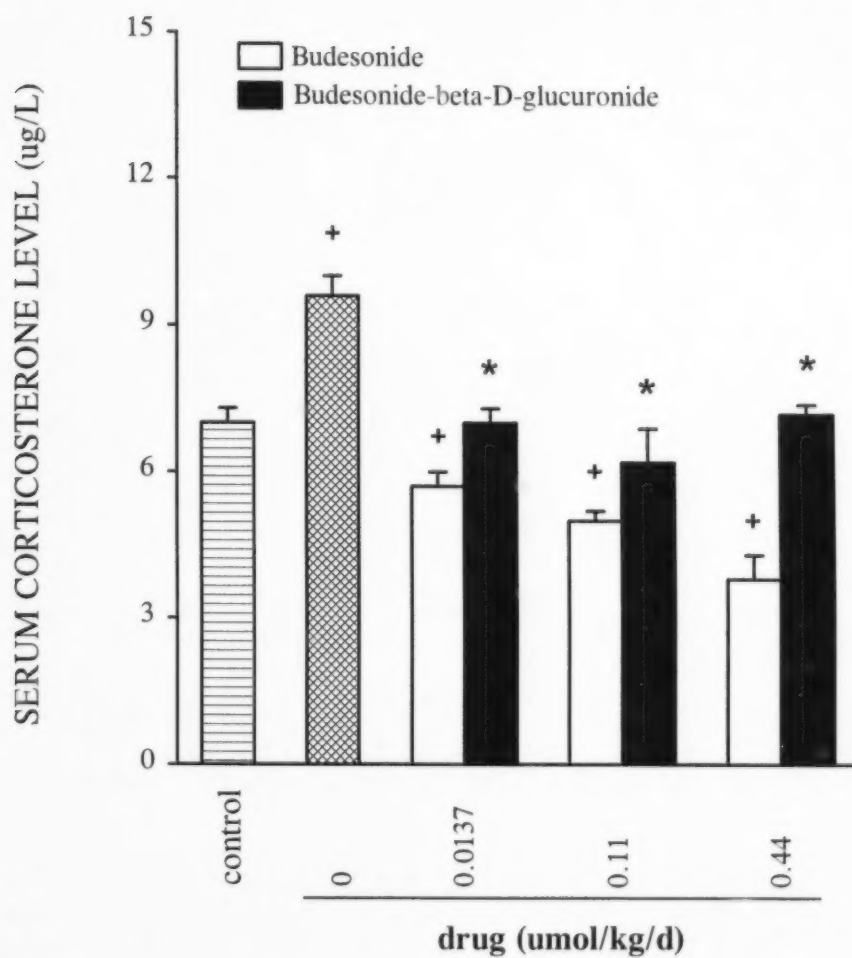


Figure 5-6. Effects of budesonide and its glucuronide prodrug on serum corticosterone level in colitic rats (n=6).

* $p < 0.05$ compared with free budesonide.

⁺ $p < 0.02$ compared with control.

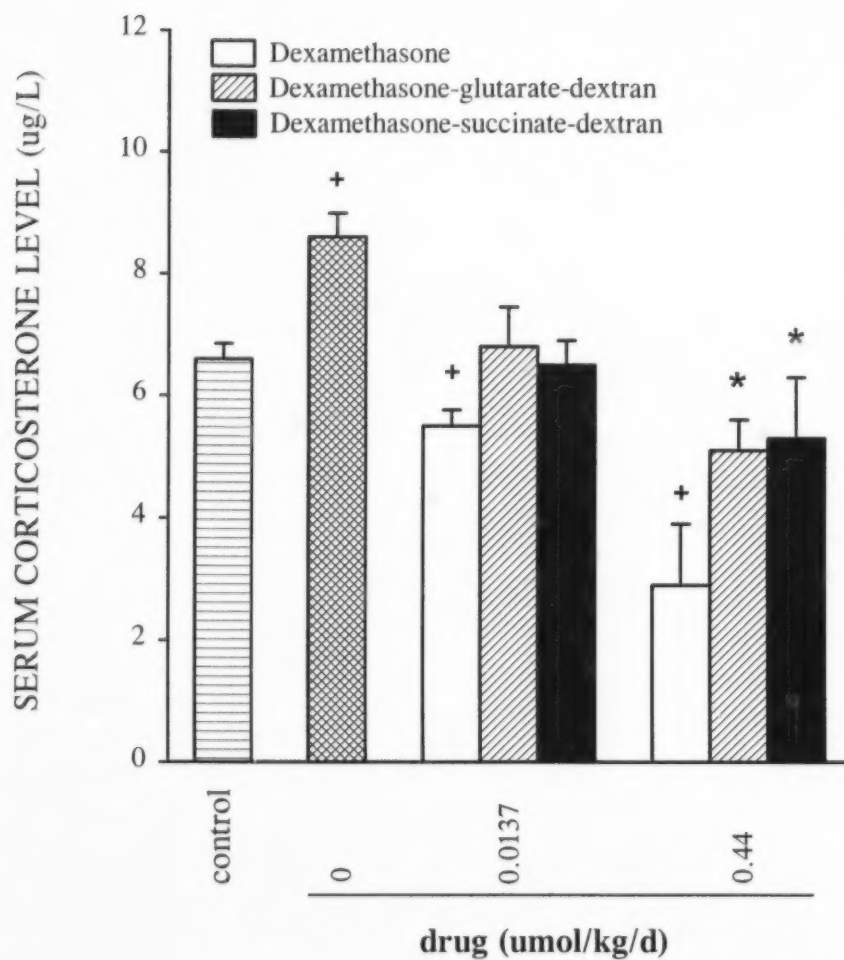


Figure 5-7. Effects of dexamethasone and its dextran prodrugs on serum corticosterone level in colitic rats (n=6).

* $p < 0.02$ compared with free dexamethasone.

⁺ $p < 0.05$ compared with control.

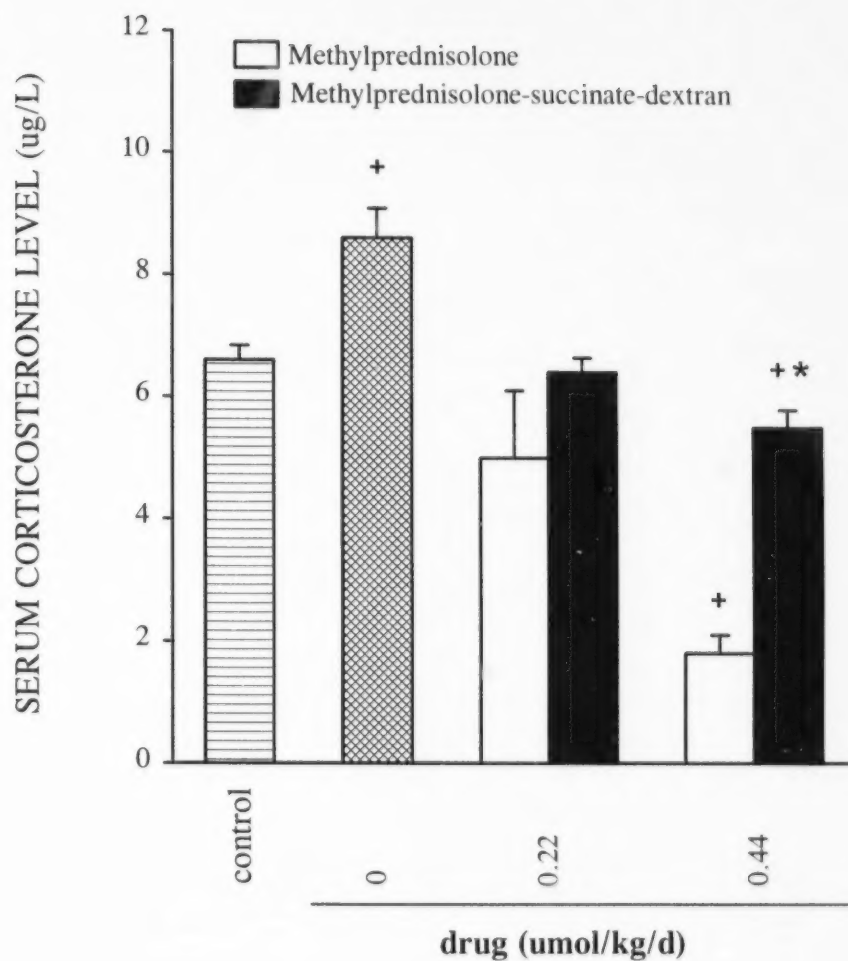


Figure 5-8. Effects of methylprednisolone and its dextran prodrug on serum corticosterone level in colitic rats ($n=6$).

* $p < 0.02$ compared with free methylprednisolone.

* $p < 0.05$ compared with control.

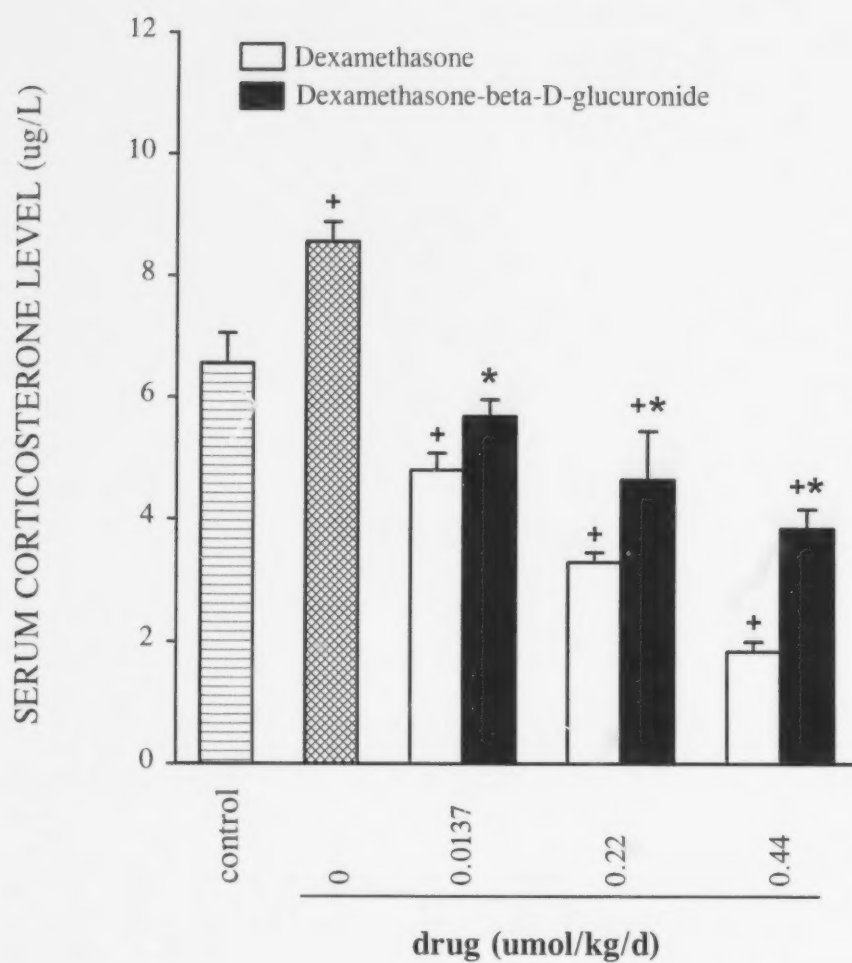


Figure 4-9. Effects of dexamethasone and its glucuronide prodrug on serum corticosterone level in colitic rats (n=6).

* p < 0.05 compared with free dexamethasone.

+ p < 0.02 compared with control.

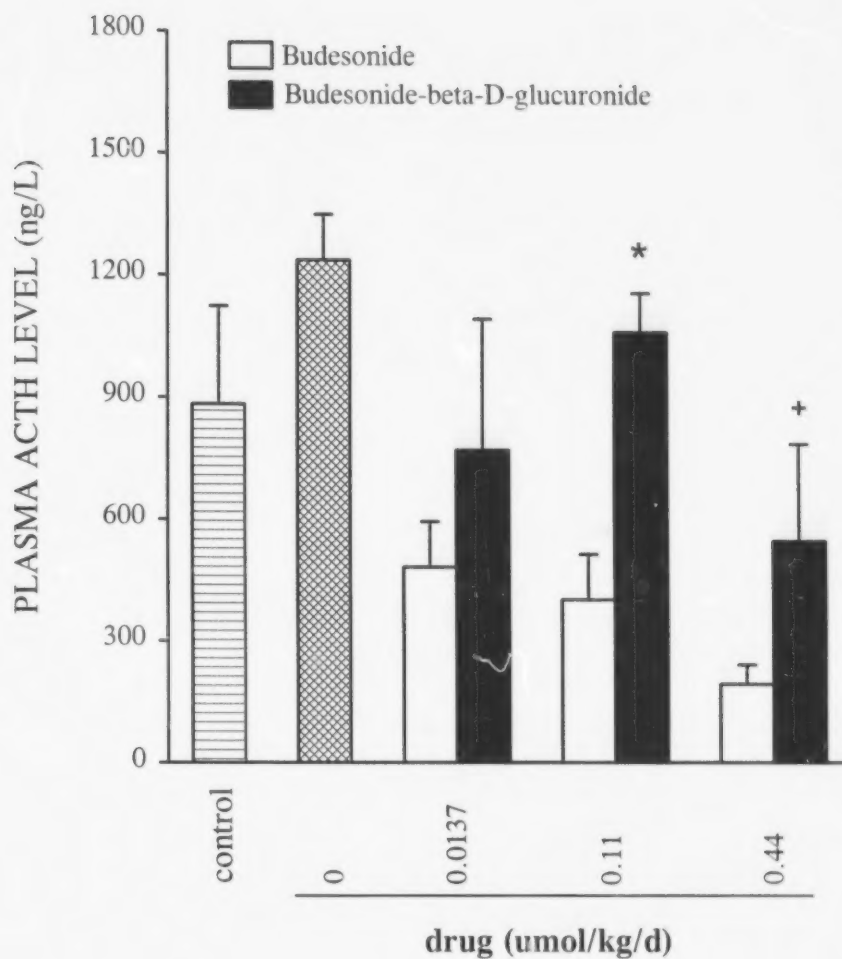


Figure 5-10. Effects of budesonide and its glucuronide prodrug on plasma ACTH level in colitic rats (n=6).

* $p < 0.02$ compared with free budesonide.

+ $p < 0.05$ compared with control.

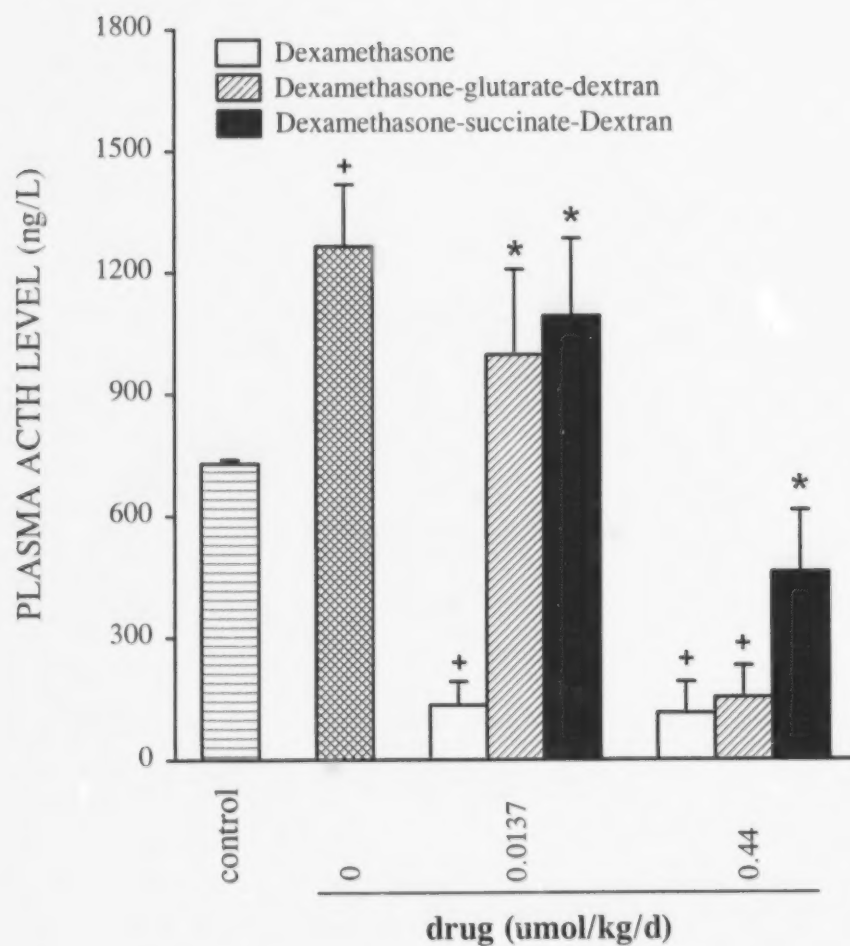


Figure 5-11. Effects of dexamethasone and its dextran prodrugs on plasma ACTH level in colitic rats (n=6).

* $p < 0.01$ compared with free dexamethasone.

+ $p < 0.01$ compared with control.

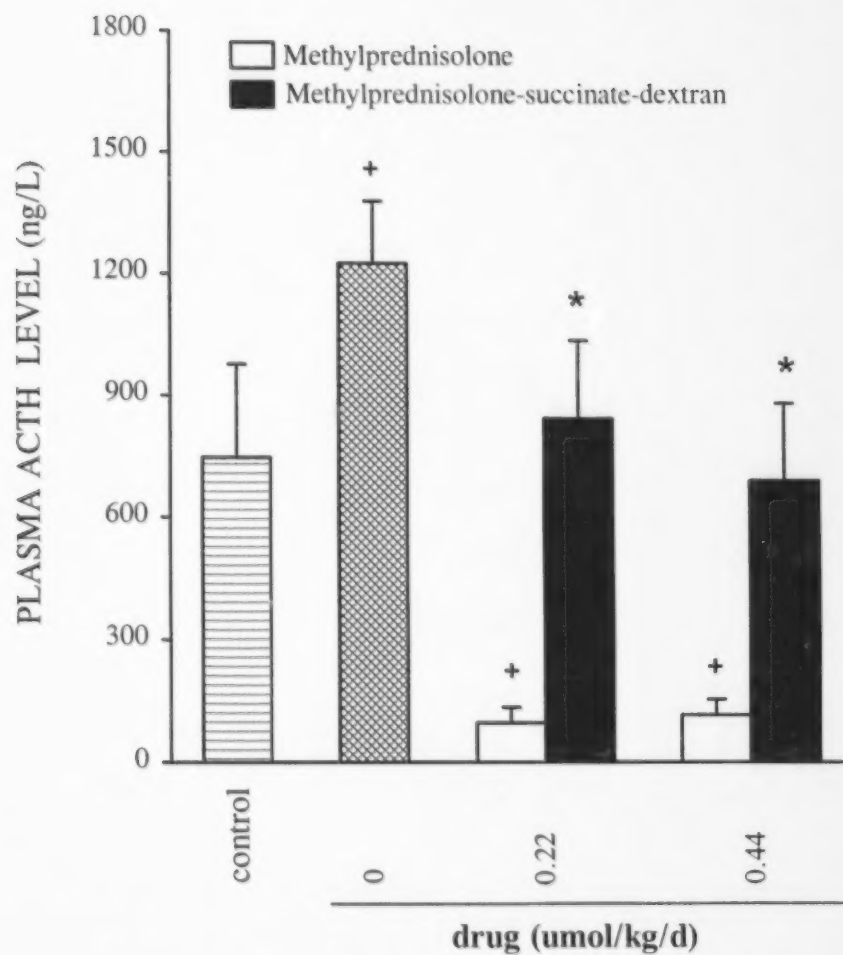


Figure 5-12. Effects of methylprednisolone and its dextran prodrug on plasma ACTH level in colitic rats (n=6).

*p<0.01 compared with free methylprednisolone.

*p<0.01 compared with control.

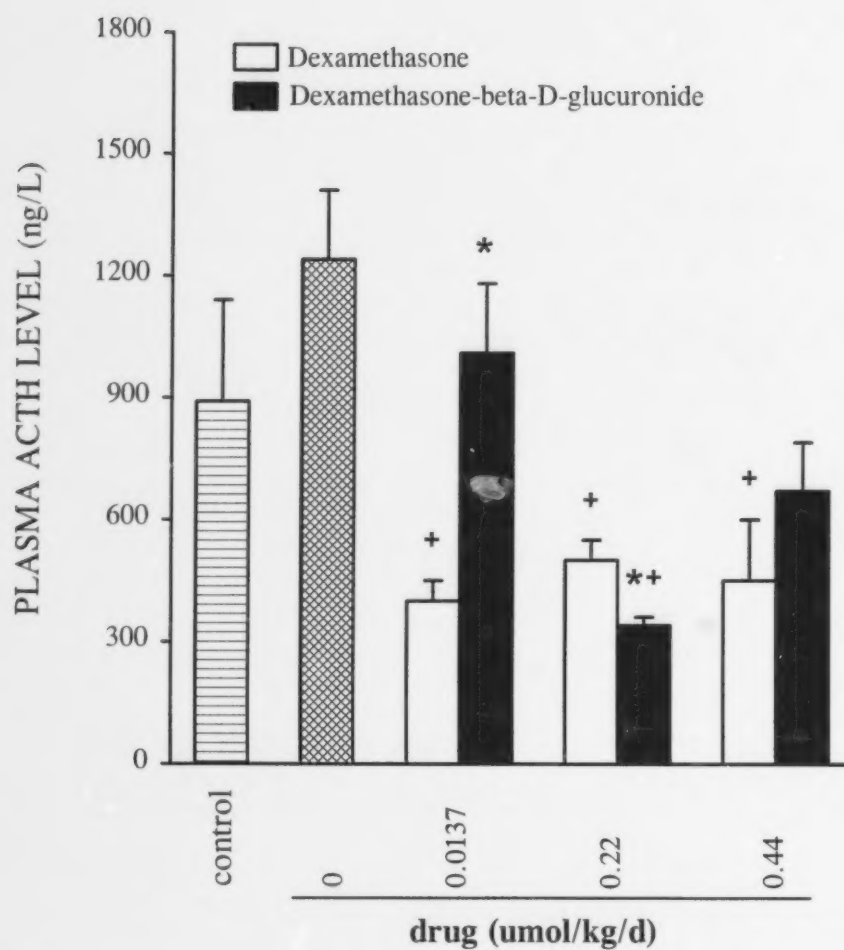


Figure 5-13. Effects of dexamethasone and its glucuronide prodrug on plasma ACTH level in colitic rats (n=6).

* $p < 0.01$ compared with free dexamethasone.

⁺ $p < 0.05$ compared with control.

CHAPTER 6

CONCLUSION

Glucocorticoids are the most powerful drugs in IBD therapy but their side-effects limit their application. In this study the stability of DX-glrd in the GI tract, and absorption of free DX into the blood stream were examined. In addition to DX-glrd, a number of other newly-synthesized glucocorticoid prodrugs (DG-dextran, DS-dextran, MPS-dextran, and BD-glrd) were tested to determine their efficacy and adrenal suppressive effects in an acetic acid-induced rat colitis model.

The results from these experiments demonstrate that DX-glrd was very stable in the small intestine and free dexamethasone was only be released in the colon. This is in agreement with the finding that the activity of β -D-glucuronidase is very low in proximal and distal small intestine and very high in the cecum and colon. Accordingly, free DX concentration in the blood stream was 3-fold higher in rats treated with free DX than in rats treated with DX-glrd.

In the efficacy studies it was found that in terms of improvement of fluid absorption function and ulcer healing, all the dextran and β -D-glucuronide prodrugs were more efficacious (from 3-16 fold) than their parent free glucocorticoids when compared by ED_{50} . Generally, the efficacy of a prodrug is determined by both the intrinsic activity of the drug and the chemical structure of the attached, inert moiety. When a free drug is combined with different carriers to form different prodrugs, the efficacy of each of the prodrugs is determined by the nature of the link between the free drug and the attached moiety. This can be seen in dextran prodrugs such as DS-dextran which has a shorter hydrocarbon chain, showed higher efficacy than DG-dextran. When different free drugs are combined with the same carrier, the free drug with the highest intrinsic activity yields the highest efficacy. Thus DS-dextran had higher anti-inflammatory activity than did MPS-dextran. Although free DX is more potent than free MP, DG-dextran did not show any obvious advantage over MPS-dextran. This can be explained on the bases that MPS-

dextran released more free drug than DG-dextran due to a shorter chain of succinic acid. Efficacy studies also showed a dose-dependent relationship. No significant differences of MPO activity were found between any of the prodrugs and its free parent drug. However, This may have been due to the timing of the treatment. Both prodrugs and free drugs were administered 24 hours after induction of colitis at which time a substantial amount of neutrophils had already infiltrated into local tissue. There was a dose-dependent relationship in reducing MPO activity and this may be a combination of the numerous effects of glucocorticoids such as the inhibition of neutrophil migration and stabilization of lysosomal membranes of neutrophils. Overall, the more potent BD prodrugs did not exhibit higher efficacy than DX prodrugs. With some prodrugs, the efficacy in fluid absorption improvement was not consistent with their efficacy in ulcer healing. These findings remain to be explained later.

In adrenal suppression studies all the dextran and β -D-glucuronide prodrugs had less adrenal suppressive effects than did their parent free drugs. Usually, the free drugs suppressed serum corticosterone and plasma ACTH at a low dose but the prodrugs did not suppress blood corticosterone and ACTH except at the highest dose. It appeared that there was not a dose-dependency of adrenal suppressive effect from more potent glucocorticoids.

The distribution of DX-glrd, anti-inflammatory efficacy and adrenal suppressive effects of DX-glrd and a number of other newly synthesized colon-specific glucocorticoid prodrugs in rat colitis model, have yielded encouraging results. Colon-specific delivery of prodrugs may be a better way to deliver active agents to local inflamed tissue, and to exert the maximum anti-inflammatory effect without adrenal suppression. After completion of animal experiments, clinical trials should be given consideration. The development of colon-specific glucocorticoid prodrugs represents a promising new therapeutic approach for the treatment of human IBD.